CHEMICAL STUDIES ON BIOTIN-DEFICIENT YEAST

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Fazal Ahmad, M.Sc.

Department of Applied Biochemistry,

Heriot-Watt College, Edinburgh

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INTRODUCTION

BIOCHEMISTRY OF BIOTIN

In the past few years many workers have tried to locate the exact loci of biotin involvement in cell metabolism but they have not been able to establish the specific functions of biotin with certainty. Numerous direct as well as indirect functions have been assigned to biotin, but, before going any further into our present knowledge of its metabolic functions, it will be pertinent to discuss briefly the history and chemistry of this vitamin.

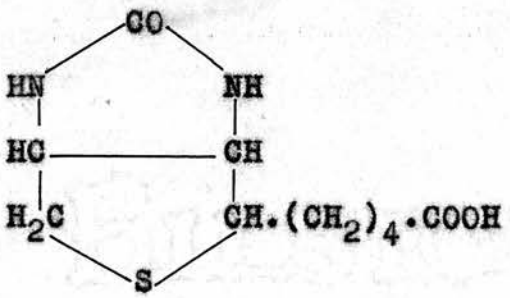
Wildiers (1901) first described the stimulating effects of small amounts of organic matter on the growth of yeasts. Wildiers also gave the name "bios" to this compound (or compounds) which caused an increase in yeast growth. Later on, "bios" was found to be multiple in nature and was fractionated into Bios I - precipitated by lead acetate, Bios IIa, unadsorbed on charcoal and Bios IIb, adsorbed on charcoal; it is this last fraction which is of concern in the present argument.

a) Chemistry of biotin: Kögl (1935) isolated in crystalline form a compound from egg yolk, which was adsorbed by charcoal, and possessed the major part of the yeast growth activity of the Bios IIb fraction. He named this compound biotin. The

empirical formula of this compound, which was in the form of its methyl ester, was provided by Kögl (1937).

Allison, Hoover and Burk (1933) obtained an extract from various sources which promoted growth and respiration of a legume nodule organism, Rhizobium trifolii, and which they named "coenzyme R". Similarly a "protective factor X" against egg white injury was reported by Boas (1927), the physical and chemical properties of which were studied by György and his associates, who called it "Vitamin H".

The identity of all of these factors was suggested by György, Melville, Burk and du Vigneaud (1940) when it was shown that biotin, coenzyme R and protective factor X possessed the same properties as a concentrate of vitamin H prepared from liver. Kögl's sample of crystalline biotin was also demonstrated to possess the properties of vitamin H by vitamin H assays (du Vigneaud, Melville, György & Rose, 1940). The formula for biotin is as given below:



(cis-hexahydro-2-oxo-1H-thieno 3,4 imidazole-4-valeric acid).

Synthesis of biotin was first reported by Harris, Wolf, Mozingo and Folkers (1943) and by Harris et al. (1944), and has since been accomplished by a number of workers in

different laboratories.

There are eight optically active modifications corresponding to the structure of biotin. Paired as racemic mixtures, these are: DL-biotin, DL-epi-biotin, DL-allobiotin and DL-epi-allobiotin. Biological activity is confined to DL-biotin. However, the racemic biotin is only half as active as the natural biotin, which is dextro-rotatory. Synthetic (D) or (+) biotin is as active as natural D-biotin, while the synthetic L-biotin is essentially inactive. The picture is further complicated by the presence of two additional isomeric forms of biotin in nature: α-biotin obtained from egg yolk (Kögl & Ham, 1943) and β-biotin obtained from liver (du Vigneaud, Hofmann, Melville & György, 1941). It has been shown for a wide variety of microorganisms (Lactobacillus casei, L. pentosus 124-2, Saccharomyces cerevisiae Y-30, Clostridium acetobutylicum S-9 and Neurospora crassa 1-A) that these isomers have identical biological activity (Krueger & Peterson, 1948).

Throughout this thesis, the term biotin will be used for β-biotin represented by the formula given on page 2.

Another important form of biotin which can be extracted from a number of sources is biotin-l-sulphoxide (Melville, Genghof & Lee, 1954). Bowden and Peterson (1949) have shown that compounds of biotin chemically combined with proteins are probably common cellular constituents. Complexes of biotin with amino acids have been synthesized

and extracted from biological materials by Wright, Cresson, Liebert and Skeggs (1952, a). The most important of these naturally-occurring complexes is called biocytin (ε-N-biotinyl-L-lysine). This was isolated from yeast extract by Wright et al. (1952, b) and can replace biotin in the nutrition of Saccharomyces carlsbergensis, but not apparently in certain Lactobacillus species. It has further been suggested that biocytin could be the predominant form of biotin in some yeast strains (Wright et al., 1952, b).

Not all biotin analogues (oxybiotin, desthiobiotin, biotin-l-sulphoxide and biocytin) possess similar biological activities. Some are not used by micro-organisms and some have anti-biotin activity. Oxybiotin and biocytin can replace biotin in the nutrition of Lactobacillus casei; biotin-l-sulphoxide is not used, while desthiobiotin is inhibitory. These analogues seem to satisfy the biotin requirement of a number of yeast species.

Dittmer, Melville and du Vigneaud (1944) and Dittmer and du Vigneaud (1947) presented evidence for the conversion of desthiobiotin to biotin by Saccharomyces cerevisiae, and it is commonly accepted that this analogue is a metabolic precursor of biotin. Pimelic acid has likewise been shown to stimulate biotin synthesis in Aspergillus niger (Eakin & Eakin, 1942) and this straight chain fatty acid has also been assigned the role of a precursor. However, to date, the ability of pimelic acid to obviate the biotin requirement

in yeasts has not been studied. More detailed information about the relationship between fatty acids and biotin is given later in this introduction.

The metabolic roles of many vitamins and growth factors have been elucidated, especially those vitamins belonging to group B. The fact that these vitamins occur as indispensable constituents of all living matter establishes their vital importance (Williams, 1945). These vitamins have also been shown to be integral parts of biological catalytic systems and are present as essential factors in the metabolic machinery of widely diverse forms (Williams, Eakin, Beerstecher & Shive, 1950). Moreover, it is known that many B vitamins act as coenzymes (Lichstein, 1958).

b) Metabolic functions of biotin: Extremely small quantities of biotin are required by living organisms for normal growth as compared to the amounts of other vitamins and growth factors. It is rather surprising therefore, that, although biotin is known to affect considerably the diverse metabolic machinery of numerous organisms, yet its exact involvement in such reactions was until recently obscure. However, a few reactions have been studied in which direct involvement of biotin has been suggested.

Mention of these reactions will be made later in this introduction, but still the picture emerging is confusing and often contradictory. It is also noteworthy that the evidence so far accumulated necessitates the assumption that biotin functions in many diverse metabolic processes some of which do not seem to share anything in common. This situation, if unexplained, leaves this member of the vitamin B family in a unique category, although inositol, which needs an extensive probe as to its modes of action, probably shares this mysterious nature with biotin.

i) Biotin and carbon dioxide fixation: Of the several metabolic functions and coenzymic roles assigned to biotin, the most important is the function that has been assigned in carboxylation and decarboxylation reactions. Thus Pilgrim, Axelrod, and Elvehjem (1942) observed a decrease in pyruvate oxidation under conditions of biotin deficiency; this is probably the result of faulty carbon dioxide transfer in the absence of biotin (Burk & Winzler, 1943). Similarly, Wessman and Werkman (1950) showed the involvement of biotin in the Wood-Werkman reaction by the use of C^{13} in Micrococcus lysodeikticus. Biotin involvement in this reaction was further evidenced by the inhibition of the reaction on addition of avidin, the inhibition being reversed when adequate amounts of biotin were added.

Similarly Ochoa, Mehler, Blanchard, Jukes, Hoffmann and Regan (1947) working with turkeys discovered a decrease in the activity of the enzyme which decarboxylates oxaloacetic acid but were unable to find an effect of biotin on other enzymes; the purified enzyme did not contain any biotin. The oxaloacetate decarboxylase from Azotobacter vinelandii was purified 40-80 times by Plaut and Lardy (1949). The biotin content of this preparation did not increase with the increase in enzymic activity. Earlier Summerson, Lee and Partridge (1941) and Pilgrim et al. (1942) observed that pyruvate was probably not utilised by rat liver slices and homogenates under conditions of biotin deficiency. Similarly Olson and his associates (1948) showed a decreased rate of pyruvate and succinate metabolism by the heart ventricular tissue when biotin was present in subnormal concentrations. The activities were restored by addition of biotin in vivo but not in vitro.

The major advance in this work on biotin involvement in CO_2 metabolism has come from the work of Wakil (1958) and Wakil et al. (1958, 1959) and Lynen and his colleagues (1959), who have demonstrated the part played by biotin in certain carboxylation reactions. Wakil and co-workers have studied the enzymes in avian liver which catalyse the transformation of acetyl-Co A to palmitic acid. Evidence has been presented for a malonic acid derivative as an intermediate in this reaction. The carboxylation of

acetyl-Co A to the malonyl derivative was catalysed by a biotin-containing enzyme in the presence of adenosine triphosphate (ATP), manganese ions and CO_2. The presence of biotin in the enzyme was confirmed by microbiological assay; also the idea of functional participation of biotin was supported by the evidence that avidin greatly suppressed incorporation of acetyl-Co A into palmitic acid. Recently Wakil and Ganguly (1959) have presented data which suggest that fatty acid synthesis proceeds as follows:

$$\text{ATP} + CO_2 + \text{acetyl-Co A} \xrightarrow[\text{enzyme}]{Mn^{++},\ \text{biotin}} \text{ADP} + \text{inorganic phosphate } (P_i) + \text{malonyl-Co A.}$$

Malonyl-Co A is readily converted to palmitate in the presence of other enzymes and co-factors.

This work furnishes evidence as to the requirement for biotin in certain carboxylation reactions. In addition, Lynen and co-workers (1959) have investigated the carboxylation of β-methyl crotonyl-Co A to β-methyl glutaconyl-Co A by cell-free extracts of Mycobacterium. It has been shown that β-methyl crotonyl-Co A carboxylase contains biotin and that the vitamin is the active group of the enzyme. The presence of biotin was demonstrated by the fact that the acid hydrolysate of the enzyme promotes growth of a biotin-requiring yeast. It was further supported by the observation that, on purification, the enzyme activity was proportional to the amount of biotin present. Inhibition of the enzyme by

avidin and the reversal of this inhibition by addition of a further quantity of biotin also contributed to the above evidence. The nature of the active CO_2 intermediate involved in the carboxylation was studied by the tracer technique. The sequence of reactions was found to be:-

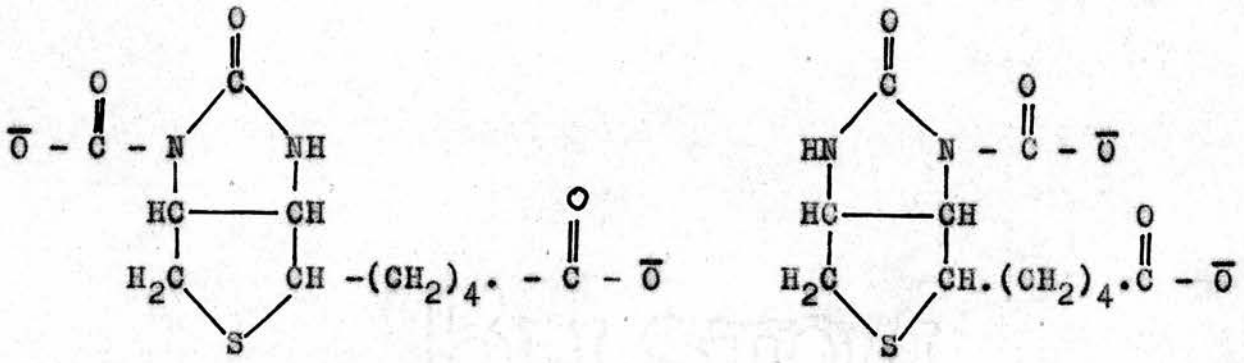
ATP + biotin-enzyme $\xrightleftharpoons{Mg^{++}}$ ADP~biotin-enzyme + P_i

ADP~biotin-enzyme + CO_2 $\xrightleftharpoons{Mg^{++}}$ CO_2~biotin-enzyme + ADP

CO_2~biotin-enzyme + β-methyl-crotonyl-Co A $\rightleftharpoons$

biotin-enzyme + β-methyl-glutaconyl-Co A.

Biotin was further found to substitute for this reaction, and the structure of the biotin~CO_2 compound proposed is as follows:-



The dimethyl ester of the enzymically prepared biotin~CO_2 complex was shown by carrier dilution and paper chromatographic techniques to be identical with the chemically synthesized carbo-methoxy biotin-methyl ester. It has further been suggested that there may be many biotin-containing enzymes.

Whitaker and Umbreit (1961) have obtained evidence supporting Lynen's hypothesis and have discovered that the activity of the ATP$\rightleftharpoons$Phosphate exchange enzyme from acetone-dried powders of Saccharomyces cerevisiae is directly proportional to the biotin content of the enzyme preparation. The activity however, was neither inhibited nor enhanced by addition of biotin. Inconsistent results were obtained on addition of CO_2, which according to Lynen's hypothesis should lower the ATP exchange. Similarly Lane and Halenz (1960) have shown that propionyl carboxylase can catalyse the ATP-independent transcarboxylation reaction probably via an enzyme-biotin-CO_2 intermediate as shown:

methyl-malonyl Co A + enzyme-biotin $\rightleftharpoons$ propionyl-Co A + enzyme-biotin-CO_2

enzyme-biotin-CO_2 + butyryl-Co A $\rightleftharpoons$ ethyl-malonyl-Co A + enzyme-biotin

Participation of biotin in the carboxylation of propionate was first observed by Lardy and Adler (1956). The same workers reported restoration of enzymic activity only in vivo, while Kosow and Lane (1961) have verified these observations and have also provided evidence for the restoration of the enzymic activity of extracts from mitochondrial acetone powders of rats caused by biotin deficiency in vivo and in vitro. Attempted reversal of the enzymic lesion in cell-free liver systems was unsuccessful.

Traub (1959) has put forward the hypothesis that the biological activity of biotin may be dependent on the formation of an intramolecular hydrogen bond between the keto-oxygen of the ureido ring and one of the carboxyl oxygen atoms of the side chain. The suggestion, that the formation of a hydrogen bond could possibly alter the charge distribution in the ureido ring system, and displace the keto-enol equilibrium to enol thus resulting in a change of chemical reactivity at the nitrogen atoms, gives support to the findings of Lynen et al. (1959), since biotin has been shown as a CO_2 carrier through a carboxylation reaction on one of these nitrogen atoms. This concept of hydrogen transport as a mode of action of biotin has also been proposed by Lichstein (1951).

The involvement of biotin in other transcarboxylation and carboxylation reactions has been reported by Swick and Wood (1960) and Stadtman, Overath, Eggerer and Lynen (1960). Lichstein (1957) found biotin in oxaloacetate carboxylase from chicken livers and reported an increase in the biotin content on purification of the enzyme. A similar figure for the amount of bound biotin in oxaloacetate carboxylase has also been given by Briggs (1960).

From the literature it appears that, although several workers have reported the exact point of biotin action in certain carboxylation, transcarboxylation and decarboxylation reactions, nevertheless many examples can be cited

where similar types of reaction have been found to be biotin-independent.

Formica and Brady (1959) studied a similar reaction to that studied by Wakil *et al.* (*loc. cit.*) *i.e.* the primary carboxylation of acetyl-Co A to malonyl-Co A in extracts of pig heart. The synthesis is quite similar to that studied by Wakil and his colleagues, but the reaction was not inhibited by avidin. Similarly Tietz and Ochoa (1959) purified 500-fold a propionyl-Co A carboxylase from pig heart extracts and obtained no evidence for the presence of biotin in the purified carboxylase or the occurrence of a separate enzymic step for CO_2 activation. No biotin could be detected after hydrolysis of the acetoacetic decarboxylase obtained in crystalline form from *Clostridium acetobutylicum* by Hamilton and Westheimer (1959). Chambers and Delwiche (1954) failed to locate the action of biotin when studying the decarboxylation of succinic acid to propionic acid in *Propionibacterium pentosaceum*. Lichstein (1958) stressed that biotin deficiency decreased the ability of *P. pentosaceum* both to decarboxylate succinic acid and to ferment glucose. Under these conditions, glucose fermentation was restored by addition of biotin, desthiobiotin or oxybiotin to the medium. Oxybiotin was found to stimulate the decarboxylation feebly while biotin and desthiobiotin were equally effective in restoring the decarboxylation ability. It was suggested that this might be due to the formation of

an oxybiotin coenzyme which has poor ability to serve as a co-factor for the succinate decarboxylase system but is fully capable of replacing the biotin coenzyme in the fermentation of glucose. The interesting suggestion was put forward that there might be many co-enzymic forms of biotin, as there are of nicotinamide and riboflavin. Woessner, Bacchawat and Coon (1958) measured the activities in the livers of biotin-deficient chicks of the two enzymes required for the carboxylation of β-hydroxy isovaleryl-Co A and found that the activity of the CO_2-activating enzyme was unchanged, whereas β-hydroxy isovaleryl-Co A carboxylase was completely lacking. It was therefore, suggested that biotin was not concerned with CO_2 fixation but with carboxyl transfer.

Since the process of carbon dioxide transfer is of fundamental importance in the metabolism of living cells, it is to be expected that any impairment in this process will bring about a major metabolic disturbance in the cell. Biotin-requiring metabolic reactions involving carbon dioxide have already been discussed above in some detail. At this stage it will not be out of place to discuss briefly those reactions in which an indirect influence of biotin has been suggested.

ii) Biotin and fatty acid metabolism: The work of Wakil mentioned earlier provided evidence for a role for biotin in fatty acid synthesis. However, the biochemical basis for the ability of certain fatty acids to spare biotin in micro-organisms is not yet fully understood.

Williams and Fieger (1946, 1947) observed an interference in the estimation of biotin microbiologically; this interference was traced to the presence of fatty acids in the samples. Subsequently it was shown that oleic acid possessed biotin-sparing activity for the assay organism, Lactobacillus casei. The biotin-sparing activity of oleic acid was later found with other micro-organisms: Lactobacillus arabinosus (Axelrod, Mitz & Hofmann, 1948; Broquist & Snell, 1951), Leuconostoc mesenteroides (Carlson, Whiteside-Carlson & Kospetos, 1950), Clostridium butyricum, Streptococcus faecalis and L. fermenti (Broquist & Snell, 1951). Similarly Lein and Lein (1949) showed the requirement of unsaturated fatty acids for a biotin-requiring mutant of Neurospora. Mathieson (1950) found that oleic acid replaced biotin in the nutrition of Ophiostoma piri and Ferguson and Lichstein (1957) extended this observation to Escherichia coli. Some saturated fatty acids (palmitic and stearic acids), though inactive in themselves, have been reported to stimulate the biotin-sparing activity of oleic acid in L. arabinosus and Clostridium butyricum

(Axelrod *et al.*, 1948; Broquist & Snell, 1951). The mechanism which controls their action is not yet known. Other unsaturated fatty acids can also replace biotin and mention can be made of lactobacillic acid which substitutes for biotin in the nutrition of *L. arabinosus* and *L. delbruckii*. Tweens, esterified with oleic acid, are claimed to be more active as growth promoting agents than is oleic acid itself (Williams, Broquist & Snell, 1947). The biotin-sparing activity of saturated cyclopropane-ring fatty acids depends upon chain length; if the chain length is less than 11 carbon atoms, not only does the acid not have biotin-sparing activity but it may even exert an inhibitory effect on certain bacilli.

The biotin-sparing activity of oleic acid has been attributed to the following:

a) The presence of a carboxyl group is necessary, for the substitution of the carboxyl group by an alcoholic group has been reported to remove activity (Axelrod *et al.*, 1948).

b) Only *cis*-isomers of oleic acid possess biotin-sparing activity (Cheng, Greenberg & Deuel, 1951; Melnick & Deuel, 1954), while the *trans*-acids are inactive. An exception is provided by elaidic acid, which is reported to be as active as oleic acid. The interesting observation made by Cheng *et al.* (1951) and Melnick and Deuel (1954) that, when biotin is present in

the medium of L. arabinosus the trans-acids were used as well, suggested that biotin may be involved in the conversion of either trans-acids to cis-acids or in increasing the absorption of trans-acids by micro-organisms. The poly unsaturated fatty acids (linoleic and linolenic acids) possess almost the same biotin-sparing activity as oleic acid for Clostridium butyricum and Streptococcus faecalis (Broquist & Snell, 1951). However, the power to replace biotin in the nutrition of L. arabinosus seems to decrease with an increase in number of double bonds (Axelrod et al., 1948; Cheng et al., 1951).

The results of the above reported investigations suggest therefore that biotin functions directly in the synthesis of oleic acid, and this is now generally accepted in explaining the biotin-sparing action of oleic acid. Efforts to prove that oleic acid acts instead as a precursor of biotin have proved fruitless (Williams & Fieger, 1946). In addition, the data presented in support of the suggestion that oleic acid acts as a cell permeability factor in enhancing uptake of nutrients by micro-organisms is neither extensive nor conclusive (Hofmann & Panos, 1954). Yet another suggestion is that oleic acid perhaps helps in the penetration of traces of biotin which may be required for normal growth in the presence of unsaturated fatty acids (Traub & Lichstein, 1956). But this idea is not in conformity with the observations of Broquist and Snell (1953)

and Williams and Fieger (1947) that avidin does not affect the biotin-sparing activity of oleic acid in micro-organisms.

iii) Biotin and amino acid metabolism: Amino acids which are the building blocks of proteins (including enzymes) are synthesized in the cell mainly by a series of amination, transamination and transdeamination reactions. It is also known that pyruvic, α-oxo-glutaric and oxaloacetic acids play an important role in acting as starting materials for many amino acid syntheses. Mention has already been made of the involvement of biotin in carboxylation and decarboxylation reactions concerning these α-oxo-acids. Biotin has also been thought to be implicated in reactions leading to amino acid synthesis. Thus Lichstein and Umbreit (1947) and Lichstein (1951) showed biotin to be essential for the deamination of aspartic acid in micro-organisms. Deamination of serine was also impaired under conditions of biotin deficiency in micro-organisms (Lichstein, 1951). The same reaction was reported to be diminished *in vitro* in liver homogenates from biotin-deficient rats. Synthesis of serine by addition of CO_2 to ethanolamine is considerably decreased under conditions of biotin deficiency; the synthesis could be restored *in vitro* but not *in vivo* (Nadkarni & Sreenivasan, 1957). Similarly Lichstein and

Umbreit (1947) and Lichstein and Christman (1948) suggested a role for biotin in the deamination of threonine. Deamination of leucine to acetoacetate has also been shown to be impaired under conditions of biotin deficiency (Plaut, 1951; Fischer, 1955). Rossi, Rossi and Rossi (1957) have demonstrated that biotin is required for the transamination processes including the glutamate-pyruvate and aspartate-α-oxo-glutarate reactions.

Of special interest is the role of biotin in aspartic acid synthesis. It was first shown by Koser, Wright and Dorfman (1942) that aspartic acid could replace partially the biotin requirement in Torula cremoris (Candida pseudotropicalis) and from this observation it was deduced that biotin is concerned directly or indirectly in the synthesis of aspartic acid. The biotin-sparing action of amino acids has also been demonstrated for a strain of Saccharomyces cerevisiae by Moat and Emmons (1954). It was shown that aspartic acid caused the most significant stimulation of growth of biotin-deficient yeast, but in addition various combinations of amino acids had stimulatory effects either in the presence or absence of aspartic acid. Further insight into the nature of the biotin requirement in aspartate synthesis was obtained by Winzler, Burk and du Vigneaud (1944). These workers presented data for biotin involvement in aspartate synthesis and suggested that the final amination is a biotin-requiring reaction.

In 1947, Lardy, Potter and Elvehjem found that oxaloacetate was capable of promoting growth of Lactobacillus arabinosus 17-5 in media deficient in both biotin and aspartic acid. These workers concluded that failure of biotin-deficient organisms to synthesize aspartate lies in the failure to condense pyruvate and carbon dioxide to oxaloacetate. The sparing action of aspartic acid on the biotin requirement for growth was shown for various species of bacteria by Potter and Elvehjem (1948). The biotin requirement of bacteria for aspartate synthesis was reported to be ten times as great as that for other functions of biotin. Later, Lardy and his associates (1949) demonstrated that biotin-deficient cells of Lactobacillus arabinosus could not fix $C^{14}O_2$ while cells grown in adequate biotin fixed $C^{14}O_2$ into cellular aspartate from $HC^{14}O_3$. Aspartate was found to contain all of C^{14} assimilated from $HC^{14}O_3$. Bettrex-Galland (1959) studied the fixation of $C^{14}O_2$ into the free amino acids of avian liver homogenates from normal and biotin-deficient animals. Aspartic acid was found to contain the major part of the radioactivity. Evidence was presented that the activities of the two enzymes, oxaloacetate decarboxylase and the malic enzyme necessary for CO_2 fixation into aspartic acid, were diminished under conditions of biotin deficiency.

The association of biotin with the synthesis of citrulline from ornithine was first reported by MacLeod

and Lardy (1949) in liver homogenates from biotin-deficient rats. Loss of ability to effect this transformation was restored by addition of N-carbamylglutamate but not by biotin (MacLeod, Grisolia, Cohen & Lardy, 1949; Feldott & Lardy, 1951). Biotin was thought to act as a catalytic factor necessary for the synthesis of carbamyl phosphate (Jones, Spector & Lipmann, 1955; Lowenstein & Cohen, 1956) which reacts with ornithine to form citrulline. Estes, Ravel and Shive (1956) reported similar results using cell-free extracts obtained from Streptococcus lactis. This impairment could not be restored in vitro by the addition of biotin, but, when biotin was added to a mixture of amino acids and other nutrients, then cells after incubation in this medium for about two hours re-established the rate of citrulline synthesis. However, a deeper probe into this problem has led Ravel et al. (1959) to suggest that biotin is not a component of the transcarbamylase but rather it exerts its action in the synthesis of this enzyme. This conclusion is supported by the finding that only very minute quantities of biotin are liberated from this purified enzyme on hydrolysis. Recently Ravel, Humpherys and Shive (1961) have shown the lack of an effect of biotin deficiency on the production of the carbamyl phosphate synthesizing enzyme of S. lactis, showing that biotin is not involved directly in the synthesis of this enzyme. The ability of this vitamin to

act as a co-factor is ruled out by the inability of avidin to affect the activity and also by the discovery that increasing activity of the enzyme on purification is accompanied by a decline in the amount of bound biotin. Thus, the exact role of biotin in citrulline synthesis remains unexplained.

iv) Biotin and protein metabolism: Several workers have suggested that biotin acts in some way in the synthesis of enzyme proteins (e.g. Blanchard, Korkes, del Campillo and Ochoa, 1950); but the mechanism of its action is incompletely understood. Poznanskaya (1957) for example showed that synthesis of pancreatic amylase and serum albumin is greatly diminished in biotin-deficient chicken tissues. The synthesis of both of these proteins was restored on injection of biotin to deficient chicks. Alpha-oxo-glutaric acid completely restored the capacity of the tissue to synthesize amylase. Similarly the capacity of the biotin-deficient liver slices to synthesize serum albumin was restored by the addition of glutamine and asparagine, which are reported to be converted readily to their oxo-acids. These results have been claimed by Poznanskaya (1957) to indicate that biotin is not involved directly in the synthesis of

proteins, and the above noted derangement has been ascribed to an alteration in the tricarboxylic acid cycle. Compensation of this derangement in the tricarboxylic acid cycle by oxo-glutarate and its precursors has been attributed to the restoration of oxidative phosphorylation by the oxo-glutarate in biotin-deficient tissues, i.e. the formation of adenosine triphosphate (ATP) which is necessary as the energy source for synthesis of protein.

Konikova, Kritsman and Yukhnovskaya (1950) reported that excess biotin ingested by biotin-deficient rats resulted in increased total protein synthesis. In a more recent investigation, these authors (Kritsman et al., 1953) concluded that biotin increased the rate of S^{35}-methionine incorporation into the body proteins. Andrew and Guerrant (1959) have tried to repeat these observations in Lactobacillus casei and L. arabinosus. The data obtained do not indicate that a biotin concentration in the medium in excess of that required for optimum growth of organism exerts any positive effect on protein formation. On the contrary, a slight negative effect was noted when L. casei was employed as the test organism.

Several other examples could be cited in which biotin has been reported to be concerned, in some indirect way, with synthesis of proteins. But in none of these has the precise function of the vitamin in protein synthesis yet been made clear.

v) Biotin and nucleotide metabolism: Several instances have been reported of an association between biotin deficiency and nucleotide metabolism. Katsuki (1959, a & b) reported the accumulation of pyruvic and α-oxo-glutaric acids in Piricularia oryzae and Bacillus macerans under conditions of biotin deficiency, while dimethyl pyruvic acid was found to be accumulated only by Piricularia oryzae. This abnormality was attributed to low contents of ATP and diphosphopyridine nucleotide (DPN), both of which are known to be essential co-factors for the successful oxidation of α-oxo-acids. Rose and Nickerson (1956) found that appreciable amounts of nicotinic acid were secreted into the medium by several yeasts under conditions of biotin deficiency. Desamido DPN (nicotinic acid adenine dinucleotide) in addition to nicotinic acid was shown to be excreted by Saccharomyces cerevisiae under the same conditions. Addition of adenine caused a diminution in the amounts of nicotinic acid and desamido DPN excreted; DL-aspartic acid up to a concentration of 0.5×10^{-3} M also brought about a decrease in the amounts excreted but higher concentrations were found to stimulate excretion of desamido DPN. These observations were taken to indicate that biotin deficiency caused a block in the synthesis of adenine which is reflected in the derangement of the synthesis of pyridine nucleotides (Rose, 1960, a & b).

It is now well established that biotin is involved in

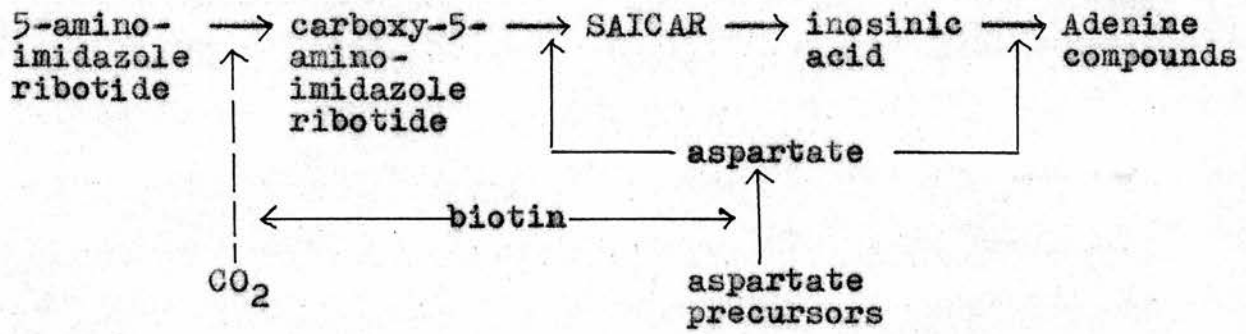
the synthesis of purines and related compounds. Thus, Cutts and Rainbow (1950) observed the formation of pink pigment by a strain of Saccharomyces cerevisiae (yeast 47), when grown under conditions of biotin deficiency and excess methionine. The accumulation of a diazotizable amine was also reported along with the pink pigment. This observation was in conformity with that of Lindegren and Lindegren (1947) who associated the pigment formation with the interaction of methionine and some precursor of adenine. Further data on arylamine accumulation was provided by Chamberlain, Cutts and Rainbow (1952) and Chamberlain and Rainbow (1954) who found that arylamine was produced by adenine- or biotin-deficient yeast. Similar observations on the accumulation of arylamine and pigmentation were later made by Moat, Wilkins and Friedman (1956). Later, evidence was provided by both groups of workers that the arylamine accumulated by yeast 47 and Saccharomyces cerevisiae 139 was 5-amino-imidazole riboside (Lones et al., 1958; Woodward & Rainbow, 1961; Friedman & Moat, 1958) rather than the free amino-imidazole. Hypoxanthine (Lones et al., 1958) was reported to be accumulated in addition to 5-amino-imidazole under conditions of biotin deficiency, although Friedman and Moat found inosine, instead of hypoxanthine to be accumulated in Saccharomyces cerevisiae 139, under the same conditions.

The link between purines and 5-amino-imidazole ribotide as their possible precursor comes from the work of

Love and Gots (1955). These workers found that a purine-requiring mutant of Escherichia coli also accumulated an arylamine, which was reported to differ from previously described intermediates. Another purine-requiring mutant transformed the new compound to 5-amino-4-imidazolecarboxamide. Chemical and physical characteristics led these workers to suggest that the accumulated product was amino-imidazole or its riboside. Amino-imida-zole and amino-imidazole carboxylic acid were reported to be produced (Rabinowitz & Barker, 1956) by the degradation of purine by Clostridium acidi-urici. It was therefore suggested that these compounds or their derivatives were possibly intermediates in both dissimilatory and assimilatory pathways. Supporting evidence came from studies by Levenberg & Buchanan (1956; 1957, a & b), who demonstrated the formation of 5-amino-imidazole ribonucleotide from formyl-glycinamidine ribonucleotide in the pigeon liver pathway. Carbon dioxide, ATP, aspartic acid and tetrahydrofolic acid were required to convert 5-amino-imidazole ribotide to inosinic acid. The reactions were taking place at ribotide level (Lukens & Buchanan, 1959).

The observations that aspartic acid (Lones et al., 1958; Friedman & Moat, 1958) depressed the accumulation of 5-amino-imidazole riboside and hypoxanthine in yeast suggested that aspartic acid is necessary for the formation of the succinyl intermediate leading to purine synthesis.

However, the increasing evidence that biotin is involved in carbon dioxide fixation into purines (Lardy & Peanasky, 1953); Wahba, Ravel & Shive, 1954) has suggested a possible role for biotin in the carboxylation of 5-amino-imidazole ribotide. There is evidence too, that the carboxylation of 5-amino-imidazole ribotide in biotin-deficient yeast requires biotin, aspartic acid and bi-carbonate (Moat & Nasuti, 1960). The scheme given below, presented by Woodward and Rainbow (1961), illustrates the possible blocks in purine synthesis caused by biotin:



SAICAR: 5-amino-4-imidazole- (N-succinylo-) carboxamide.

It is apparent that biotin is controlling three reactions - two through its relationship to aspartic acid synthesis, and the third through its role in carbon dioxide fixation.

Synthesis of riboflavin by Aspergillus oryzae is considerably attenuated by biotin deficiency. The normal rate of synthesis is restored on addition of biotin, here again biotin is probably implicated in the incorporation of carbon dioxide in the synthesis of purine bases which are

known precursors of riboflavin (Tirunarajanan et al., 1954).

Aspartic acid is involved in the biosynthesis not only of purines but also of pyrimidines. The results of Lagerkvist, Reichard and Ehrensvard (1951) and Reichard and Lagerkvist (1953) confirm and amplify those of previous investigators and, together with isotopic and microbiological evidence, clearly implicate aspartic acid as the donor of N-3; C-4; C-5 and C-6 of the pyrimidine ring. The N-1 of the pyrimidine ring comes from ammonia (Lagerkvist, 1953), while C-2 has been shown to be contributed by CO_2 (Heinrich & Wilson, 1950). The indirect involvement of biotin in the production of yet another major constituent of nucleic acids seems once again to be through its role in the biosynthesis of aspartic acid.

In the light of the above evidence, one would expect a derangement in the synthesis of nucleobases under conditions of biotin deficiency, which most likely would be reflected in a decreased total nucleic acid content. Contradictory reports are found concerning the influence of biotin on the content of nucleic acids in living organisms. Superoptimal concentrations of biotin in the medium of Lactobacillus arabinosus, L. casei and Bacterium subtilis have been reported to decrease the synthesis of ribo- and deoxyribonucleic acids, perhaps causing a disorder in the processes leading to nucleotide synthesis (Gothoskar, Rege & Sreenivasan, 1954). Contrary to this

observation, biotin deficiency has been shown to increase slightly the content of ribo- and deoxyribonucleic acids in L. casei (Prusoff, Teply & King, 1948). Similarly Cooper and Brown (1958) observed a diminution in ribonucleic acid content in the cytoplasm of the liver, heart and kidneys of new-born rats from biotin-deficient mothers while the deoxyribonucleic acid content was unchanged.

CHEMICAL COMPOSITION OF YEAST

Several of the early microbiologists made analyses of the overall chemical composition of yeasts but, as the various structures and organelles in living cells were discovered, so attention began to be focussed on the chemical composition of these structures. It is, therefore, more desirable to discuss the chemical anatomy of Saccharomyces cerevisiae, when attempting to relate chemical composition to metabolic activity.

a) Cell wall: The pioneer work of Dawson (1949) first made the investigation of microbial cell walls a possibility. He showed that shaking suspensions of bacteria

with small glass beads in a sonic vibrator resulted in the separation of cell walls from cells so that it was possible to isolate the cell wall fraction and study the walls in a fairly clean condition. Walls from a wide variety of micro-organisms have now been isolated and studied. The composition of the yeast cell wall, obtained by disrupting the cells in a Mickle disintegrator, has been studied by Northcote and Horne (1952), Kreger (1954) and Roelofsen and Hoette (1951). Examination of the cell wall preparations under the electron microscope showed that they consisted of at least two shells (Northcote & Horne, 1952). After the removal of lipids, it was found that extraction of mannan and protein with dilute alkali left one shell consisting of glucan. Glycogen, which had frequently been found to accompany mannan and glucan in wall preparations following dissolution of whole cells by means of strong alkali, is probably not a constituent of the cell wall proper. Northcote and Horne were subsequently able to isolate glycogen from mechanically disrupted cells of yeast in the form of a particulate fraction, quite separate from the cell wall residues. Bartholomew and Levin (1955) and Agar and Douglas (1955) conducted experiments on extremely thin sections and were able to demonstrate the multiple nature of the cell wall membranes. Some workers, concluded that the two shell structure originally proposed for the yeast cell wall was

probably an over-simplification. Falcone and Nickerson (1956) have isolated a protein-mannan complex from the cell wall of baker's yeast. More detailed studies of the nature of the yeast cell wall have revealed the presence of a glucan-protein and two gluco-mannan-protein complexes Kessler & Nickerson, 1959). Of particular interest is the report that the two gluco-mannan-protein complexes in cell wall of Candida albicans were found to be rich in biotin (Nickerson, 1961).

Northcote and Horne (1952) and Hurst (1952) determined the lipid content of the cell walls of baker's yeast and found it to be 8.5% by weight. The lipid mainly consisted of neutral fat. This figure compares with one of 4% (w/w) reported for the lipid content of whole cells of Saccharomyces cerevisiae (Newman & Anderson, 1933), which suggests that lipid is probably concentrated in the cell wall.

Many workers have reported evidence for the presence of enzymes in or on the yeast cell wall. Thus, Wilkes and Palmer (1932) inferred from their observations that invertase was in the outer region of the yeast cell. This view was further substantiated by the work of Demis, Rothstein and Meier (1954). Similarly enzymes, capable of hydrolysing adenosine triphosphate or glucose phosphates, have been shown to be present in the region of the yeast cell wall or at the surface of the plasma membrane (Rothstein & Meier, 1949). Sevag, Newcomb and

Miller (1954) reported the *in vivo* inactivation of α-glycero-phosphatase by the anti-enzyme serum. Because of the size of this antibody molecule, it was assumed that the anti-enzyme has access only to the surface of the cell wall; hence the presence of another enzyme was associated with the surface of the cell.

The porosity of the cell wall is shown by the fact that low molecular weight substances can penetrate this region. The size of the pores seems to be fairly small, since larger molecules, such as gelatin, inulin and polypeptides are unable to penetrate into the cell. From studies on yeast cell walls, it appears that these walls are fairly rigid and resistant to chemical agents. It is fair to assume that the principal function of the cell wall is to serve as a tight-fitting structure, confining the protoplast and preventing it from undergoing osmotic lysis, even when (as is commonly the case) the osmotic pressure of the medium is lower than that of the cell contents. Certain properties of yeasts suggest that the extreme outermost surface possesses an organised structure, but little at present is known concerning this.

b) Protoplast: This is the term reserved for the organised protoplasmic elements of microbial cells deprived completely of the cell-wall structure. Formation of naked protoplasts

of micro-organisms has been carried out by dissolving away the cell wall in high osmotic pressure medium, using reagents such as lysozyme, ethylenediamine tetraacetic acid (EDTA) and penicillin. Yeasts, however, are resistant to these reagents, although it has recently been shown that protoplasts of Saccharomyces cerevisiae can be prepared using an enzyme extract from snail gut (Eddy & Williamson, 1959). Yeast protoplasts are bounded by a plasma membrane, inside which are several discrete structures or organelles in a complex colloidal matrix. It would appear that no information is yet available concerning the chemical composition of the plasma membrane in yeast. However, Weibull and Bergstorm (1958), have shown that the plasma membrane in bacteria consists mainly of phospholipid and protein complexes. In the following paragraphs, there is a brief discussion of the composition and structure of the more important of the organelles in yeast.

i) Nucleus: Interpretations of cytological observations on the nucleus and chromosomes in yeast are at present widely divergent. This is perhaps a reflection of the comparatively small size of the yeast nucleus (the diameter of the stained chromatin in a resting nucleus is only about 0.5 - 1.5μ). Also, during the early investigations, confusion about the distribution of deoxyribonucleic acid

(DNA), which occurs as a conjugated protein, arose, because the amount of DNA present in yeast is relatively small in comparison with the total nucleic acid. A method for extracting DNA from yeast was described by Chargaff and Zamenhof (1948) and so the presence of this important cell constituent was established. In recent years, several other workers have demonstrated the presence of DNA in yeast and evidence is now available which suggests that the role of DNA is as important in yeast as in other living cells. The purine and pyrimidine bases which occur in DNA are the same as in ribonucleic acid (RNA), except that uracil is replaced by thymine. The nature of the sugar in DNA also differs from that in RNA and is recognised to be D-2-deoxy-ribose.

The physical properties of the DNA obtained from yeast, such as viscosity, dispersing power and the diffusion coefficient, strongly suggest that the molecule is very large. The molecular weight of a comparatively undegraded preparation of DNA is of the order of 1.0×10^6.

The nucleus, which chemically consists of deoxyribo-nucleoprotein, is made up of genes and chromosomes, recognised as the agents transmitting heredity characteristics. The role of specific proteins or enzymes in the control of metabolic reactions in living cells is well established and clearly, therefore, DNA must control the synthesis of these specific proteins. In the past few decades several workers,

by their outstanding efforts, have demonstrated an indirect rather than a direct control of DNA on protein synthesis. There is evidence, too, that the indirect role of DNA in protein synthesis is through ribonucleic acid (RNA). It is thought that DNA exerts its control over protein synthesis by directing the synthesis of specifically distinct RNA molecules, which in turn act as templates or moulds in protein synthesis. While little is known concerning the biochemical relationships between DNA and RNA synthesis, there are many indications that the nucleus is the RNA-forming site (Mazia, 1956; Goldstein & Plaut, 1955), or that the presence of DNA is necessary for RNA synthesis.

ii) Cytoplasmic particles: The presence of particulate elements in yeast cytoplasm has long been recognised by direct microscopic examination of both stained and unstained preparations. Cytological studies by Yotsuyanagi (1955) showed at least two different kinds of particles in addition to inclusions of fat and glycogen, which had been noted by previous workers, and volutin granules, which are probably composed of polyphosphates (Lindegren, 1947). Glycogen and trehalose are the two reserve polysaccharides in yeasts. The percentage compositions of these two carbohydrates vary considerably in different strains of yeasts. Strains of Saccharomyces cerevisiae usually contain less lipid ($<7\%$)

than other yeasts, the quantity varying with the strain and with the conditions under which the yeast is grown. While much of this lipid would seem to be in the cell wall, that in the cytoplasm occurs as lipid granules.

The particles observed by Yotsuyanagi (1955) belong to two groups. The first group are visible as highly refractile granules and stain with dilute alcoholic solutions of Sudan III. The nature and significance of these granules has yet to be elucidated. The second type are known as mitochondria. These are probably identical with particles isolated by Linnane and Still (1955) from baker's yeast after centrifugation at 25,000 g. This preparation was active and oxidised various substrates involved in the tricarboxylic acid cycle, consuming oxygen, in the process. This was the first isolation of respiring mitochondria. Particulate materials, containing cytochromes a, b and a little c, have been prepared by Chantrenne (1953). These particles are only visible in the ultramicroscope and probably represent wholly, or in part, a degraded fraction of the mitochondria. Analysis of a similar preparation showed it to contain lipo-protein containing lipid (24%) and phosphorus (1.3%), one-third of the latter being in the form of RNA.

Until recently reliable evidence for the presence in yeast cytoplasm of ribosomes (microsomes, consisting of ribonucleoprotein and membranous material composed of lipids)

was lacking owing to the extremely small diameter (~0.1 μ.) of these particles as compared with ribosomes from plant and animal cells. However, more recently, Bowen et al. (1961) have reported the nature of ribosomes present in yeast. Also, the base composition of the soluble and ribosomal RNA from baker's yeast has been elucidated (Monier et al., 1960).

iii) Cell sap: The cell sap is a complex mixture of substances varying from simple inorganic ions to extremely complex but soluble organic molecules. These substances include, water, vitamins (free and as co-factors), amino acids (free and in the combined form), lipids, nitrogenous bases for nucleic acids synthesis, RNA and proteins (mostly enzymic in nature). Cell constituents are synthesized from these relatively simple molecules by the anabolic reactions which take place during growth of micro-organisms.

The most important of the cell constituents with regard to the present study are RNA and proteins. Reference has already been made as to the involvement of RNA and DNA in protein synthesis.

The present study was undertaken in order to study the effects of biotin deficiency on the synthesis of nucleic acids and protein by a strain of Saccharomyces cerevisiae.

The work was extended to follow the effects of various biotin-sparing substances on the synthesis of these cell constituents, and a study was also made on the influence of biotin on the activities of certain enzymes. It was hoped that this study might throw some light on the role of biotin in the metabolism of Saccharomyces cerevisiae. Certain of the preliminary work reported in this thesis has already been published and a copy of this publication is appended.

METHODS

CULTURAL METHODS

a) Organism: The biotin-requiring strain of Saccharomyces cerevisiae (Fleishmann 139) used throughout this study was obtained from Miss M.T. Clement of the Division of Applied Biology, National Research Council of Canada, Ottawa. It was maintained on malt-wort agar: 10% (w/v) spray dried malt extract ("Muntona", Munton and Fison Ltd., Stowmarket, Suffolk) + 2% (w/v) agar. Fresh slope cultures were prepared each month by transferring a portion of the material from the old slope. After streaking, the slopes were incubated at 25° for 24-48 hr. Cultures so obtained were stored at 3°. There was no routine micro-biological examination of the yeast during this period of study, but its constancy of behaviour during growth in various defined media strongly suggested that the stock cultures had remained genotypically unchanged.

b) Medium: The chemically defined medium of Rose and Nickerson (1956) was used for the growth of the organism. The complete list of ingredients included in the medium is given hereunder:

COMPOUND:	Concentration/litre
Glucose (A.R.)	20.0 g.
Ammonium sulphate (A.R.)	3.0 g.
Potassium dihydrogen orthophosphate (A.R.)	3.0 g.
Calcium chloride 6 H_2O (B.D.H.)	0.25 g.
Magnesium sulphate 7 H_2O (B.D.H.)	0.25 g.
i-Inositol (L. Light and Co. Ltd.)	10.0 mg.
Calcium-D-pantothenate (L. Light and Co. Ltd.)	1.0 mg.
Thiamin hydrochloride (L. Light and Co. Ltd.)	1.0 mg.
Pyridoxin hydrochloride (L. Light and Co. Ltd.)	1.0 mg.

D-Biotin was incorporated in this medium as described under RESULTS, usually in either a suboptimal concentration (0.4×10^{-10} M) or a concentration optimal (8.0×10^{-10} M) for growth of the yeast.

Other compounds were also included in the medium as described under RESULTS. With the exception of oleic acid (B.D.H.) and vitamin free casamino acids (Difco Laboratories Inc.), all of these compounds were obtained from L. Light and Co. Ltd. Gas chromatographic analysis of the sample of oleic acid used showed it to contain:

C_{12}	saturated	fatty acids	0.5%
C_{14}	"	"	0.5 - 1.0%
C_{15}	"	"	trace
C_{16}	mono-unsaturated	"	4.0 - 7.0%
C_{16}	saturated	"	2.5 - 5.0%
C_{17}	mono-saturated	"	1.0 - 1.5%

C_{18}	di-unsaturated (linoleic)	fatty acids	2.5 - 3.5%
C_{18}	tri-unsaturated (linolenic)	"	0.8 - 1.3%
C_{18}	saturated	"	1.0 - 2.0%
C_{19}	mono-unsaturated	"	0.5 - 1.0%
C_{18}	mono-unsaturated (oleic)	"	63.0 - 76.0%
C_{18}	" (elaidic)	"	18.0 - 22.0%

Compounds tested for biotin-sparing activity were screened for possible contamination with biotin using the test organism. Batches found to be contaminated were rejected.

It may also be pointed out that, in medium completely deprived of exogenous biotin, there was no measurable growth of the yeast for at least 72 hr. during incubation. The slight increase in turbidity after this time was probably a reflection of the synthesis of biotin by the organisms. It was therefore necessary to supply some minimum quantity of biotin to ensure the start of growth.

Portions (100 ml.) of the medium were dispensed into 350 ml. conical flasks. Culture flasks were washed by soaking in 4% (w/v) sodium hydroxide in 75% ethanol overnight, neutralising the excess alkali with dilute acetic acid, washing with tap water and rinsing thrice with distilled water; the flasks were allowed to drain over filter papers and were then dried in an oven at 140-150°. The same washing procedure was used for other glassware,

except graduated apparatus which was dried by draining only. Flasks were plugged with non-absorbent medical grade cotton wool and autoclaved momentarily at 10 lbs./sq.in.

Samco tubes were also used for testing the biological purity of the compounds employed in this study, and for examining the ability of individual or combinations of compounds to spare biotin. The use of Samco tubes has been described by Northam and Norris (1952). Only tubes with internal diameters between 1.625-1.675 cm. were used.

c) Inoculation technique: A special technique for inoculation was called for because malt-wort contains appreciable quantities of biotin and, if this biotin were not drained out of the cells before inoculation, then it would be difficult to control the amounts of biotin in the medium. To overcome this difficulty, Rose (1960, b) devised a technique for inoculation of cultures in which the introduction of biotin was effectively minimized. This was carried out as follows:

A portion of material from a slope culture was transferred to 6.0 ml. of sterile biotin-free medium in Samco tubes and the turbidity was brought up to 0.09-0.11 mg./ml. dry weight of yeast. The tubes were incubated at 25° statically for 18 hr. during which time the dry weight of

yeast reached 0.32-0.39 mg./ml. The contents of the tubes were transferred aseptically into separate sterile 50 ml. centrifuge tubes and the supernatant fluids rejected after centrifugation. Each cell crop was washed three times with 6.0 ml. portions of sterile M/15 potassium dihydrogen orthophosphate buffer (phosphate buffer) of pH 4.5 and, finally, the washed cells were suspended in 6.0 ml. of sterile phosphate buffer; 0.6 ml. of this suspension was then added to a further 6.0 ml. of sterile phosphate buffer. This diluted suspension was then used for inoculation at the rate of 1 drop/6.0 ml. of the medium. Pasteur pipettes used for inoculation purposes were of fairly constant dimensions. Each drop was determined to be approximately equal to 30 µl.

d) Incubation: Cultures were incubated statically at 25°.

e) Harvesting of cells: Cells were harvested by centrifuging the cultures and removing the supernatant fluid in each case by decantation. Each crop was washed thrice with ample quantities of M/15 potassium dihydrogen orthophosphate buffer (pH 4.5). Each cell crop so obtained was suspended in a suitable volume of this buffer before withdrawing aliquots of cells for analyses.

f) Measurement of growth: At the end of the incubation period, cultures were shaken thoroughly and 6.0 ml. of suspension was then transferred to a Samco tube. Growth was measured turbidimetrically by determining the optical density in the Hilger 'Spekker' absorptiometer (model 760), using neutral green-grey H-508 filters against a water blank. Optical density measurements were then related to mg./ml. dry weight of yeast by a calibration curve kindly provided by Dr. Rose.

ANALYTICAL METHODS

Nucleic acids: Triplicate 3.0 mg. dry weight portions of the washed crop of yeast were taken for nucleic acids estimations.

a) Acid-soluble ultraviolet-absorbing substances: The acid-soluble ultraviolet-absorbing substances were extracted using 0.2N-perchloric acid or 5% (w/v) trichloroacetic acid. The reasons for selecting these solvents to extract the acid-soluble ultraviolet-absorbing substances are given under RESULTS (Section 1, b). The yeast was suspended into either 2.0 ml. or 4.0 ml. of the ice-cold extractant and incubated for 5 min. at 3°. The supernatant was collected after centrifugation. The process was repeated with the same volume of extractant and the supernatant fluids were either pooled or kept separate. Extracts were brought up to 5.0 ml. either by the addition of extractant or by neutralisation with an appropriate amount of alkali followed by addition of M/15 phosphate buffer (pH 4.5). The procedure used for each individual set of experiments is mentioned under RESULTS. The optical density of this

extract was measured at 260 mμ in a Unicam (Cambridge) S.P. 500 Quartz spectrophotometer against an appropriate blank and the reading was taken as a measure of the amount of acid-soluble ultraviolet-absorbing substances present in the yeast. Generally, two extractions were found to be necessary to remove completely the acid-soluble ultraviolet-absorbing substances from 3.0 mg. dry weight of the yeast.

The pellet of cell debris remaining after the extraction of the acid-soluble ultraviolet-absorbing substances was defatted by suspending it into 3.0 ml. of a boiling mixture of ethanol 95% (v/v, in water) + ether (1 vol.) for 2 min. The procedure was repeated once more and the supernatant obtained after centrifugation was rejected.

b) Ribonucleic acid: Throughout this study, a modification of Schmidt and Thannhauser's method (1945) has been used for the extraction of ribonucleic acid (RNA). The residue obtained after the removal of acid-soluble ultraviolet-absorbing substances and lipids was suspended in 2.0 ml. of N sodium hydroxide for 1 hr. at room temperature (Bonar & Duggan, 1955). Longer periods of extraction ranging from 1 hr. to 12 hr. were tried, but little difference was observed in the amounts of RNA extracted; this could presumably be explained by the fact that the small amounts

of RNA present in 3.0 mg. dry weight of yeast were easily hydrolysed to smaller polynucleotide units in this short period of time. At the end of this period, the suspension was made 0.2 N with respect to perchloric acid by addition of N-perchloric acid. This procedure led to the precipitation of deoxyribonucleic acid (DNA) and protein (Schmidt & Thannhauser, 1945). The residue obtained after centrifugation was washed twice with 1.0 ml. portions of 0.2 N-perchloric acid and the washings were pooled. These were then either neutralized with sodium hydroxide and made up to 10.0 ml. by addition of M/15 phosphate buffer (pH 4.5) or made directly to 10.0 ml. by addition of 0.2 N-perchloric acid. The optical density of this extract was then measured at 260 mμ. against an appropriate blank and was taken as a direct measure of the RNA content in 3.0 mg. dry weight of yeast.

c) Deoxyribonucleic acid: The deoxyribonucleic acid (DNA) was extracted by suspending the residue obtained after RNA extraction in 2.0 ml. of N-perchloric acid and holding the contents at 90° for 15 min. (Ogur & Rosen, 1950). The contents were allowed to cool and were then centrifuged. The volume of the supernatant fluid was brought up to 3.0 ml. by addition of N-perchloric acid. The optical density of

this extract at 260 mμ. was taken as a direct measure of the DNA content in 3.0 mg. dry weight of yeast.

d) Analysis of ribonucleic acid: Full practical details of the procedures used in the analysis of ribonucleic acid (RNA) are given, as no complete account is available in the literature.

Duplicate portions (40.0 mg. dry weight) of yeast were taken in 50 ml. centrifuge tubes and extracted with 10.0 ml. portions of ice cold 5% (w/v) trichloroacetic acid at 3° for 5 min. The efficiency of trichloroacetic acid in extracting this acid-soluble ultraviolet-absorbing fraction is given under RESULTS. It was necessary to eliminate the acid-soluble ultraviolet-absorbing substances completely by extracting the yeast repeatedly with ice-cold 5% (w/v) trichloroacetic acid. The optical density of the extracts was measured at 260 mμ. and the yeast was subjected to further experimentation only when this fraction had been completely removed. Occasionally 5-7 extractions with ice-cold 5% (w/v) trichloroacetic acid were required to eliminate this fraction entirely. The residue obtained was defatted as described previously.

The residue was suspended in 2.0 ml. of 0.3 N-potassium hydroxide and incubated at 37° for 18 hr. This period was

found satisfactory to degrade RNA quantitatively to mononucleotides. Stronger alkalies, e.g. N-sodium hydroxide or potassium hydroxide, were also found to bring about the hydrolysis in shorter periods, but these reagents are known to alter chemically the structure of some of the components of the RNA (Davidson & Smellie, 1952). At the end of this period, the contents were made 0.2-0.3 N with respect to perchloric acid by adding 9.2 N-perchloric acid, which treatment precipitated deoxyribonucleic acid (DNA) and protein. The supernatant fluid containing the RNA mononucleotides was removed by centrifugation and its pH was adjusted to 4.0 by careful addition of 10.0 N-potassium hydroxide. The use of potassium hydroxide for this purpose was preferred to sodium hydroxide because of the insolubility of potassium perchlorate, which was formed on neutralisation. At this stage the second sample was treated as described under The separation of nucleobases by paper chromatography. The fluid was once again centrifuged in the cold to remove remaining potassium perchlorate, the presence of which was found to interfere during paper chromatography and electrophoresis.

i) Electrophoretic Separation of Mononucleotides: The volume of supernatant containing the mononucleotides of RNA obtained from 40 mg. dry weight of yeast varied from 1.5-2.0 ml. and the desired volume was spotted directly on the paper for

electrophoresis. An aliquot of this solution (300-500 µl.) was applied on Whatman No. 3 MM paper as a short band (1.5-2.0 cm.) about 15 cm. from one end of a strip of paper (57 x 10 cm.). The paper was soaked in citrate buffer (pH 3.5) in such a manner that 1 cm. on each side of the spotted area was left dry. Excess buffer was blotted using filter paper. The paper was then placed between the glass plates of the electrophoretic apparatus described by Durrum (1950). Both ends of the paper dipped into citrate buffer. The compartments were connected to attain equilibrium and to irrigate the paper completely; this took 15-20 min. The compartments were then disconnected and water was allowed to circulate in the cooling plates to minimize evaporation during passage of the electric current. Since Durrum's apparatus and its modification could not overcome the problem of evaporation from the paper, comparatively low voltage gradients (15-20 volts/cm.) were used. The carbon electrodes immersed in the compartments containing the citrate buffer were connected to a source of D.C. power supply giving a voltage gradient of 15-20 volts/cm. Under these conditions, a period of 7-7½ hr. was sufficient to separate the mono-nucleotides. After removal, the paper was dried by hair dryer, and the nucleotides detected under ultraviolet light ('Hanovia', Chromatolite), the positions being outlined with pencil markings. The areas of paper containing the spots were cut out and the nucleotides eluted from the shredded paper

by soaking in 5.0 ml. of 0.01 N hydrochloric acid overnight at 37° in stoppered 10 ml. tubes. The contents of the tubes at the addition of the eluant and at the end of the incubation period were vigorously shaken. The eluates were centrifuged to remove cellulose fibres. The optical density of each eluate was then measured at an appropriate wavelength, using blanks prepared by removing an area of filter paper equal to that of the area containing the spot. The wavelengths used were: adenylic acid, 260 mμ; guanylic acid, 260 mμ; (Volkin & Carter, 1951), uridylic acid, 262 mμ; and cytidylic acid, 278 mμ (Ploeser & Loring, 1949).

The molar concentrations of the nucleotides were calculated from the optical density measurements using the appropriate extinction coefficients. Controls of adenylic, guanylic, cytidylic and uridylic acids were run with each electrophoretogram.

Recovery of adenylic acid after electrophoresis as described above was found to be 94-96% as calculated from optical density measurements.

A commercial preparation of yeast RNA (L. Light and Co. Ltd., Slough, Bucks.) was used as a reference sample. A suitable weight (4.0 mg.) of this preparation was suspended in 2.0 ml. of 0.3 N-potassium hydroxide, and was then treated by the procedure as detailed above.

ii) Chromatographic separation of ribonucleobases: The second sample left after extraction and hydrolysis of the ribonucleic acid to mononucleotides was acidified to precipitate deoxyribonucleic acid, protein and potassium perchlorate. The residue was washed twice with 0.5 ml. portions of 0.2 N-perchloric acid and the washings were pooled. This solution was then adjusted to pH 4.0 by careful addition of 10 N-potassium hydroxide. The contents were centrifuged in the cold to remove potassium perchlorate and the clear supernatant was heated to dryness in a boiling water bath.

Previous workers have used various acids to liberate quantitatively the nucleobases from RNA (Levene & Bass, 1931; Hotchkiss, 1948 and Vischer & Chargaff, 1948), but with conflicting results. However, treatment of yeast RNA with 12 N-perchloric acid for 1 hr. at 100° has been reported to cause no appreciable destruction of any of the nucleobases (Marshak & Vogel, 1951). Liberation of all the bases quantitatively by this treatment has been claimed by Wyatt (1951) for various RNA samples. The present author used this method extensively during the early period of this study and found it to give satisfactory results.

The dried sample in a tapered centrifuge tube was suspended in 0.2 ml. of 70-72% perchloric acid, and the contents were held in a boiling water bath for 70 min. The solution was then cooled and diluted to double its volume.

This solution was then neutralised by addition of 10 N-potassium hydroxide. Perchloric acid was found to corrode the chromatogram paper even in dilute solution; it was essential, therefore, to neutralise it. The contents of the centrifuge tube were then acidified with 12 N-hydrochloric acid to increase the solubility of the bases, warmed in a water bath for 2-4 min., cooled and centrifuged to remove potassium perchlorate and carbon. The precipitate was washed once with 0.1 N-hydrochloric acid and the supernatant fluids pooled, the total volume being 0.6-1.0 ml. The utmost care was taken to remove potassium perchlorate, since this compound was found to interfere during chromatographic separation of the bases. This supernatant fluid was then applied as a band (1.0-1.5 cm.) on Whatman No. 3 MM paper and developed by descending chromatography using the solvent of iso-propanol: + conc. hydrochloric acid: + water (68:16.4:15.6) described by Wyatt (1951). The paper was irrigated for 40 hr. at room temperature (18-20°), dried using a current of cold air, and examined under ultraviolet light. The ribonucleo-bases appeared as discrete absorbing spots, the guanine spot being easily distinguished by its bluish tinge (Smith & Markham, 1950). The spots were outlined with light pencil marks, cut out and eluted by soaking the shredded pieces in 5.0 ml. of 0.1 N-hydrochloric acid in stoppered glass tubes at 37° overnight. To allow for the ultraviolet-absorbing substances in the paper, blanks were prepared by eluting an

area equal in size and at an equal distance from the starting line. The optical densities of the eluates were measured at the appropriate wavelength against the corresponding blank; the wavelengths were:- adenine, 260 mμ; guanine, 250 mμ; cytosine, 275 mμ and uracil, 260 mμ (Wyatt, 1951). The molar concentrations of the bases were calculated by using the appropriate millimolar extinction coefficients. Controls of adenine, guanine, cytosine and uracil were run with each chromatogram. This technique was shown to give recovery values about 96% when a solution of adenine was applied on a chromatogram paper.

e) Protein estimations: Two methods were used for the estimation of protein.

Protein remaining in the residue after nucleic acids and related substances had been extracted from the yeast was estimated by the micro-Kjeldahl technique. The samples in the tapering centrifuge tubes were directly washed into digestion flasks with 1.0 ml. portions of water. The digestion flasks contained 500 mg. of potassium sulphate (A.R.), 50 mg. of mercuric oxide (Miller & Houghton, 1945), and a few grains of nitrogen-free sand. Sulphuric acid (A.R.) (2.0 ml.) was added to each centrifuge tube, which was shaken well and the acid poured into the digestion flask.

This procedure helped in complete transference of the contents into the digestion flasks. The flasks were then heated on a rack, slowly at first until the water had evaporated, which took 15-30 min. The sand was added to avoid bumping, which otherwise was difficult to control. After the removal of water, the temperature of the flask contents was raised to boiling and the contents took 15-20 min. to clear. The digestion was allowed to proceed for a further period of 5 hr. After this period, the flasks were allowed to cool, a drop of ethanol was (95% v/v) added, and the digestion was continued for a further period of 1 hr. Duplicate blanks, containing 3.0 ml. of water, were also digested.

The volumes of the digests were brought up to 10.0 ml. by adding water. Duplicate aliquots of 4.0 ml. each were separately distilled for 6 min. in an apparatus described by Markham (1942), with 4.0 ml. of sodium hydroxide (A.R.) 40% aqueous solution, containing 5% (w/v) sodium thiosulphate (A.R.). When using mercuric oxide as catalyst, it is necessary to add sodium thiosulphate to the sodium hydroxide reagent to avoid incomplete recovery of ammonia, otherwise mercury ammonium complex is not decomposed. Excess of alkali over that required was also avoided, to prevent the decomposition of mercuric sulphide (Clark, 1943) which otherwise interferes with the estimation of ammonia. Ammonia evolved was trapped in 2.0 ml. of boric acid (0.5% w/v, A.R.),

containing two drops of achromic indicator (methyl orange, methylene blue). The boric acid solution containing ammonia was back titrated against N/500 sulphuric acid, the titre being used to calculate the amount of nitrogen present in the samples. Protein contents are expressed as mg. protein/3.0 mg. dry wt. or as µg. Kjeldahl nitrogen per 1.0 mg. dry weight of yeast.

The protein content of cell-free extracts of yeast was estimated colorimetrically using the method of Lowry, Rosebrough, Farr and Randall (1951).

Cell-free extract (0.05-0.1 ml.) obtained by breaking open the yeast cells, as described below, was taken in Samco tubes. The volume of this extract was made up to 0.7 ml. with water, and this was followed by addition of 3.0 ml. of alkaline copper sulphate reagent [(0.5% (w/v) copper sulphate in 1.0% (w/v) potassium tartarate; 1.0 ml. of this mixture was diluted to 50.0 ml. by addition of 2% (w/v) sodium carbonate in 0.1 N-sodium hydroxide)]. The contents in the Samco tubes were mixed and allowed to stand at room temperature (18-20°) for 15 min. Finally 0.3 ml. of Folin-Ciocalteu (1927) reagent (B.D.H. diluted to make it N in acid) was added rapidly, the solution being mixed immediately after addition. The colour which developed was measured after ½ hr., at 500 mµ for concentrated samples or at 750 mµ for samples containing

less than 25 µg. of protein/ml. of the reaction mixture, in a Unicam S.P. 600 spectrophotometer using 1 cm. glass cells. The optical density was related to the protein concentration by a standard curve plotted using bovine serum albumin (L. Light and Co. Ltd.). Protein contents are expressed as µg. protein/ml. or as otherwise stated.

f) Intracellular amino acid pool: Water-soluble ninhydrin-positive substances were extracted from yeast, which had been washed thrice with ice-cold water, by suspending 10.0 mg. dry weight in 10.0 ml. of water and holding the contents for 10 min. in an oil bath at 140-150° (Gale, 1947). On cooling, the supernatant fluid was removed from the cell debris by centrifugation; after being supplemented with washings (2.0 ml.) from the cell debris, the volume of the extract was made up to 10.0 ml. with water. The extracts were stored at -4° if not immediately assayed. The extracts were assayed for total ninhydrin-positive substances using a modification of the method described by Smith and Agiza (1951). Duplicate 0.5-1.0 ml. portions of the extract were added to 1.0 ml. of citrate buffer (pH 5.0); 1.0 ml. of ninhydrin solution (5.5 mg./ml. in citrate buffer) and 1.0 ml. of stannous chloride solution (0.2% (w/v) in citrate buffer) were added to each tube. The tubes were placed in a boiling water bath

and kept there for 20 min.; after which time they were removed to a beaker containing crushed ice and allowed to cool in the dark for 10 min. Rapid cooling in the dark was essential to obtain consistent results. The contents of each tube were then brought up to 10.0 ml. by addition of ice-cold saturated solution of sodium chloride, and this was followed by the addition of 5.0 ml. of ice-cold n-butanol. Each tube was stoppered, vigorously shaken and allowed to stand in the dark for 5 min., during which time the blue colour was taken up in the butanol layer. This layer was removed, and the optical density of the butanol extract measured at 570 mμ in a Unicam S.P. 600 spectrophotometer, using 1 cm. glass cells; blanks were prepared using 0.5-1.0 ml. of water instead of 0.5-1.0 ml. of the extract. Optical density readings were related to μg. amino group/ml. by a standard curve prepared using purified glycine (B.D.H.). The results which are taken as a measure of the intracellular amino acid pool in the yeast, are expressed as μg. amino group/10.0 mg. dry weight of yeast.

g) Adenosine triphosphate: The determination of adenosine triphosphate (ATP) was carried out by the method of Bowen and Kerwin (1956).

Myosin B, adenosine triphosphatase (ATPase) was prepared

from rabbit muscle by a modification of the method of Györgyi (1947) as described by Bowen and Kerwin (1956). This preparation of myosin B did not require further purification, since the action of actin present as impurity in this preparation was inhibited by the use of ethylenediamine tetraacetic acid (EDTA). Actin is known to dephosphorylate adenosine diphosphate (ADP), while the activity of myosin is confined to the dephosphorylation of ATP to ADP.

Acetone-dried powders of the yeast were prepared by the method of Katsuki (1959). Yeast harvested by centrifugation was washed thrice with ice-cold water as quickly as possible. The whole pellet of yeast was then washed thrice with 30 ml. portions of ice-cold acetone (A.R.), after which treatment the yeast was spread on a filter paper and dried under vacuum for 1-2 hr. over anhydrous calcium chloride. The moisture content determined in different preparations was found to be consistently 12-14%.

For dry weight determinations, duplicate samples of acetone-dried powders (10-15 mg.) were weighed directly into 15 ml. tapered centrifuge tubes. The tubes were heated at 110° for 1 hr., cooled and one drop of water was added to each tube. The tubes were then swirled so that a thin film of yeast was formed on the sides. The tubes were again heated until a constant weight was established.

For the estimation of ATP and ½ hr. labile-phosphate (the term ½ hr. labile-phosphate has been used to account

for the colour produced due to slight acid hydrolysis of polyphosphorylated compounds extracted from the yeast, while estimating inorganic phosphate by the method of Fiscke and Subbarow (1925), aliquots (15-20 mg. dry weight) of the acetone-dried powders were weighed in 15 ml. centrifuge tubes and suspended in 2.0 ml. of boiling water (Katsuki, 1959). The tubes were placed immediately in a boiling water bath and an additional portion of 2.0 ml. of boiling water was added. The contents were well mixed and kept in the boiling water bath for 10 min. The tubes were cooled in running water and centrifuged, and the supernatant was made up to 5.0 ml. by addition of water. The pH of this extract was found to be 6.8. Duplicate 2.0 ml. portions of this extract were taken; one was subjected to dephosphorylation with myosin while the other was used directly for the determination of "½-hr. acid-labile" phosphate.

The assay mixture for the dephosphorylation of ATP consisted of 2.0 ml. of the acetone-dried powder extract obtained as mentioned above in 0.05 M tris-(hydroxymethyl aminomethane) buffer (pH 8.0), 0.1 ml. of EDTA (0.2 M), 0.8 ml. of 30% (w/v) potassium chloride, and 6.0 mg. of myosin B. The volume of the mixture was brought up to 5.0 ml. with water and the mixture incubated at 25° for ½ hr. with shaking at 15 min. The reaction was stopped at the end of this period by adding 0.5 ml. of 50% (w/v) tri-

chloroacetic acid. The contents were centrifuged to remove the precipitated myosin, and inorganic phosphate was determined in the supernatant fluid. The difference between the phosphate obtained on dephosphorylation and that determined directly was taken as a measure of ATP content.

The method described by Fiscke and Subbarow (1925), with the slight modification proposed by Bowen and Kerwin (1956), was used for the estimations of inorganic phosphate. An aliquot of the solution containing phosphate was placed in a 25 ml. measuring flask, followed by 1.5 ml. of 10 N-sulphuric acid and 2.5 ml. of 4% (w/v) ammonium molybdate. The contents were mixed, and then 0.5 ml. of the reducing reagent (a mixture of 1.2 g. sodium sulphite and 1.2 g. sodium metabisulphite + 0.2 g. 1-amino-2-naphthol-4-sulphonic acid per 100 ml. of solution) was added. The volume was brought up to the mark by addition of water. Each flask was allowed to stand for ½ hr. after shaking the contents. The extinction of this solution was measured in a Hilger 'Spekker' absorptiometer against an appropriate blank using a filter of 660 mμ. The phosphate content of each unknown was related to a standard curve plotted by using potassium dihydrogen orthophosphate (A.R.). The phosphate contents are expressed as μmoles phosphorus/g. dry weight of yeast and the ATP content as μmoles ATP/g. dry weight of yeast.

h) Preparation of cell-free extracts: Yeast (100 mg. dry weight) harvested by centrifugation, was washed thrice with ice-cold water. The organisms were then suspended in 5.0 ml. of ice-cold water and disrupted in a tissue disintegrator (H. Mickle, Gomshell, Surrey) with 3.0 g. of Ballotini beads (grade 12). The disintegrator was operated at maximum amplitude. The temperature during the breaking process was kept at 5-10° by alternate periods (5 min.) of shaking and cooling in an ice bath. Effective dissipation of heat during this process was also achieved by circulating ice-cold water around the glass cells. The period required to obtain maximum breakage varied with the nature of the yeast; this is discussed under RESULTS. Usually a ½ hr. disintegration period was found satisfactory. Each disrupted suspension was centrifuged at 4500 r.p.m. in the cold to remove the beads and cell debris. The supernatant fluid was used either immediately or within 24 hr. for estimations of various enzymes. When the extract was not used soon after its preparation, it was stored at -4° to -6° without loss of activity of the enzymes under investigation. The protein contents of the cell-free extracts were determined by the method of Lowry et al. (1951).

ENZYME ASSAYS

a) Acid pyrophosphatase: For the determination of cell-wall acid pyrophosphatase, adenosine triphosphate (ATP) was used as substrate. A suitable quantity of yeast (10.0-12.0 mg. dry weight) was harvested by centrifugation, washed thrice with ice-cold water and once with 0.1 M acetate buffer (pH 3.6). The method of Rothstein and Meier (1949), slightly modified, was used.

The reaction mixture consisted of 10.0-12.0 mg. dry weight of washed yeast, 2-4 µmoles of ATP in 0.1 M acetate buffer (pH 3.6), 5.0 µmoles of magnesium chloride, and 0.1 M acetate buffer (pH 3.6) to make a total volume of 2.0 ml. The mixture after shaking was incubated at 30° for 1 hr. with shaking at 15 min. intervals. At the end of this period, the reaction was stopped by removing the yeast by centrifugation. The clear supernatant was withdrawn and the inorganic phosphate in it was estimated by the method of Fiske and SubbaRow (1925). The amount of inorganic phosphate thus estimated was taken as a measure of the acid pyro-phosphatase activity. Appropriate blanks for the determination of non-enzymic hydrolysis of ATP and leakage of phosphate from the yeast cells were conducted simultaneously under the conditions of the experiment.

Concentrations of yeast up to 6.0 mg. dry weight/ml. gave proportional acid-pyrophosphatase activities under the conditions of the experiment.

Acid-pyrophosphatase activity is expressed as µmole ATP hydrolysed/mg. protein/hr. at 30°.

b) Invertase: Yeast (6.0 mg. dry weight) was suspended in 6.0 ml. of 5% (w/v) sucrose solution in M/15 potassium dihydrogen orthophosphate buffer (pH 4.5). After shaking, 2.0 ml. of the suspension was immediately withdrawn and inactivated by addition of 2.0 ml. of 2 N-sodium hydroxide. Yeast cells were removed by centrifugation and the supernatant fluid was used as blank. The remainder of the yeast suspension was incubated at 37° for one hr. After this period it was inactivated as described above, centrifuged, and the reducing sugar content of the supernatant estimated by the Nelson-Somogyi (1944, 1945) method. Concentrations of yeast up to 2.0 mg. dry weight/ml. gave proportional invertase activities under the conditions of the experiment.

Invertase activity in cell-free preparations was determined by taking a suitable aliquot of the extract (0.2-0.7 ml.) and bringing its volume to 6.0 ml. by addition of 5% (w/v) sucrose in M/15 phosphate buffer

(pH 4.5), followed by the procedure as detailed above.

Invertase activity is expressed as µmoles sucrose hydrolysed/mg. protein (whole cells or cell-free extract)/hr. at 37°.

Reducing sugar was determined as follows: Duplicate samples containing 50-300 µg. of reducing sugar produced by the action of invertase on sucrose were taken in 25 ml. boiling tubes. Somogyi reagent (2.0 ml.) was then added to each boiling tube. The tubes were immersed in a vigorously boiling water bath and held for 10 min., followed by immediate cooling by plunging the tubes into a cold water bath. Arsenomolybdate reagent (2.0 ml.) was added to each tube and the mixture was diluted to 25.0 ml. by addition of water. The optical density of the samples was measured at 520 mµ. in a Unicam S.P. 600 spectrophotometer using 1 cm. glass cells against appropriate blanks. The optical density of the unknown samples was related to the standard curve plotted using glucose (A.R.). Standards were run containing 50, 150 and 300 µg. of glucose per tube with every second set of determinations, but no deviation was observed from the standard curve.

c) Malic dehydrogenase: Malic dehydrogenase in cell-free preparations was estimated by a modification of the method described by Beaufay, Bendall, Baudhuin and du Duve (1959).

The assay involved following the increase in optical density at 340 mμ. in the Unicam S.P. 500 Quartz spectrophotometer in 3.0 ml. of the reaction mixture contained in a 1 cm. quartz cell. The reaction mixture consisted of 0.02 M tris buffer (pH 8.5) + mM EDTA + 0.01 M sodium cyanide + 0.7 μM of diphosphopyridine nucleotide (DPN) + 600 μM potassium-L-malate. This mixture, in which the high concentration of cyanide was necessary to trap oxaloacetate, was allowed to stand at 25° for at least 2 hr. to allow the DPN and cyanide to equilibrate. The reaction was started by addition of 0.1 - 0.5 ml. of the enzyme preparation. The quantity of the enzyme protein required to obtain stoichiometric response in the optical density varied from 50-150 μg. Two reference cells were used to follow the increase in optical density; one contained all the components of the reaction mixture except potassium L-malate, while from the other cell both enzyme preparation and DPN were excluded. The rates measured in this manner were constant up to a change in optical density of 0.1, and were found to be proportional to the concentration of enzyme. Increase in optical density was always followed in duplicate test samples contained in quartz cells. The molar extinction coefficient for reduced diphosphopyridine nucleotide (DPNH) at 340 mμ.

(6.22×10^6 cm.^{2} mole^{-1}) used was that given by Horecker and Kornberg (1948).

Enzymic activity is expressed as umoles L-malate dehydrogenated/µg. protein/min. at 25°.

d) Carbamyl phosphate ornithine carbamyl transferase (CPOC transferase): The method of Estes et al. (1956) was used for the determination of CPOC transferase activity in cell-free preparations.

For the assay, 5-20 µg. of protein contained in 0.05-0.2 ml. of the extract was incubated for 2 hr. at 30° with carbamyl phosphate (dilithium salt, L. Light & Co. Ltd., 20 µM) + magnesium chloride (A.R., 2.5 µM) + DL-ornithine hydrochloride (L. Light & Co. Ltd., 20 µM) + tris-(hydroxy)-methyl-amino-methane buffer (pH 8.0) in a total volume of 1.0 ml. The mixture was well shaken after each ½ hr. interval. At the end of this period, the reaction was stopped by addition of 2.5 ml. of N-perchloric acid. The citrulline produced was determined by the method of Archibald (1944). The enzymic activity was found to be proportional to the amount of citrulline produced over the range of 30-300 µg./ml. of the reaction mixture.

The enzymic activity is expressed as m moles ornithine reacted/mg. protein/hr. at 30°.

The citrulline content of the reaction mixture was estimated as follows:

An aliquot containing 20-100 µg. of citrulline was made up to 4.0 ml. by addition of water; 2.0 ml. of the acid mixture (1 vol. conc. sulphuric acid (A.R.) + 3 vol. syrupy orthophosphoric acid (A.R.)) was added, followed by addition of 0.25 ml. of aqueous solution of diacetyl monoxime (3.0% w/v). The contents of the tubes were well agitated and the tubes were covered with glass bulbs and heated in the dark for 15 min. in a boiling water bath. The tubes were then cooled in the dark for 10 min. with occasional and vigorous shaking. Once the tubes were heated, they were protected from light till the optical densities of the contents of the tubes were measured because of the photo-labile nature of the colour produced. The optical densities were measured at 490 mµ. in a Unicam S.P. 600 spectrophotometer. Blanks prepared from inactivated enzyme preparations were used for the optical density measurements. The optical density reading was related to the standard curve obtained by using known concentrations of citrulline (L. Light & Co. Ltd.).

RESULTS

SECTION I

EFFECT OF BIOTIN DEFICIENCY ON THE SYNTHESIS OF NUCLEIC ACIDS AND PROTEIN BY SACCHAROMYCES CEREVISIAE

a) Contents of nucleic acids, protein and related substances in biotin-deficient and biotin-optimal yeast: Initially, an examination was made of the effect of biotin deficiency on the contents of DNA, RNA, acid-soluble ultraviolet-absorbing substances, protein and intracellular amino acids in the yeast. Cultures of the yeast, grown in media containing either an optimal (8.0×10^{-10} M) or a suboptimal (0.4×10^{-10} M) concentration of biotin, were taken after various periods of incubation at 25° and, after growth had been measured, the yeast was harvested, washed and analysed for DNA, RNA, acid-soluble ultraviolet-absorbing substances, protein and intracellular amino acids. The data showing the contents of DNA, RNA, acid-soluble ultraviolet-absorbing substances and protein are given in Figs. 1 and 2, while data on the amino acid pool are given in Table I.

Under conditions of biotin deficiency, growth of the yeast was restricted and, after about 120 hr. of incubation, the biotin-deficient yeast was coloured pink instead of the usual creamy white (Chamberlain et al., 1952). This

Figures 1 & 2

Effect of incubation time on growth (●———●, mg. dry wt./ml.) and on the contents of DNA (X———X), RNA (○———○), acid-soluble ultraviolet-absorbing substances (◑-----◑) and Kjeldahl protein nitrogen (△-----△, mg./3.0 mg. dry wt. yeast x 10) in yeast grown in media containing either an optimal (8.0×10^{-10} M) (Fig. 1) or a suboptimal (0.4×10^{-10} M) concentration of biotin. Analyses were conducted on 3.0 mg. dry wt. portions of yeast. Contents of DNA, RNA and acid-soluble ultraviolet-absorbing substances are expressed as the optical densities at 260 mμ of extracts from the yeast made up to 3.0, 10.0 and 5.0 ml. respectively with appropriate extractants.

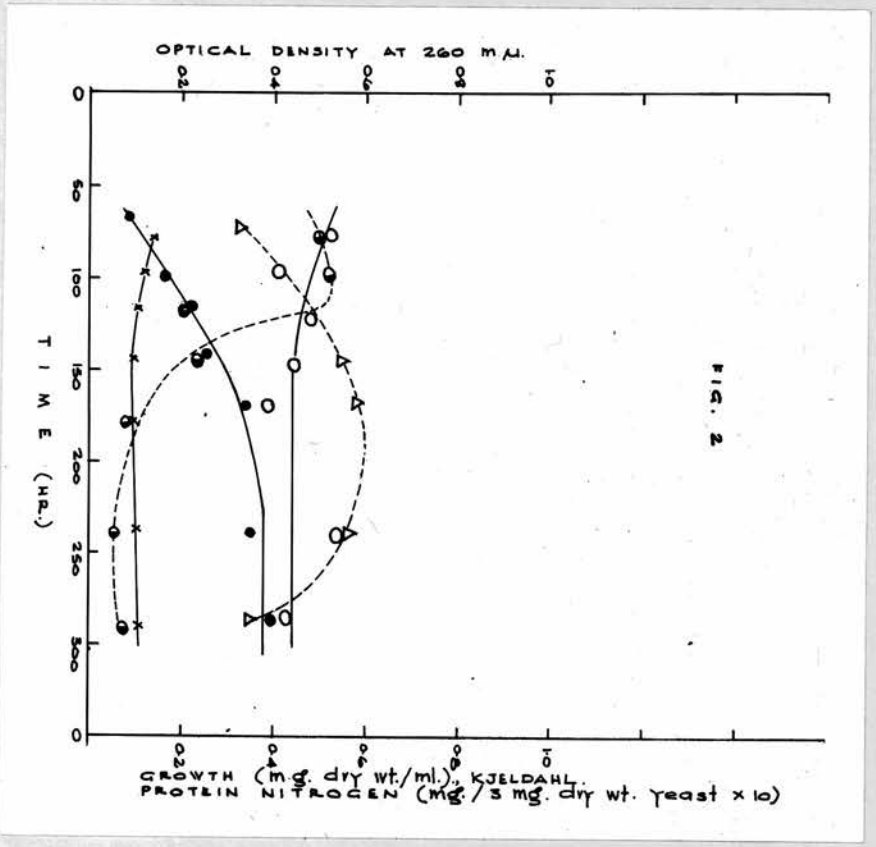

OPTICAL DENSITY AT 260 mμ.
0.2
0.4
0.6
0.8
1.0
0
50
100
150
200
250
300
TIME (HR.)
GROWTH (mg. dry wt./ml.). KJELDAHL.
PROTEIN NITROGEN (mg./3 mg. dry wt. yeast x 10)

FIG. 2

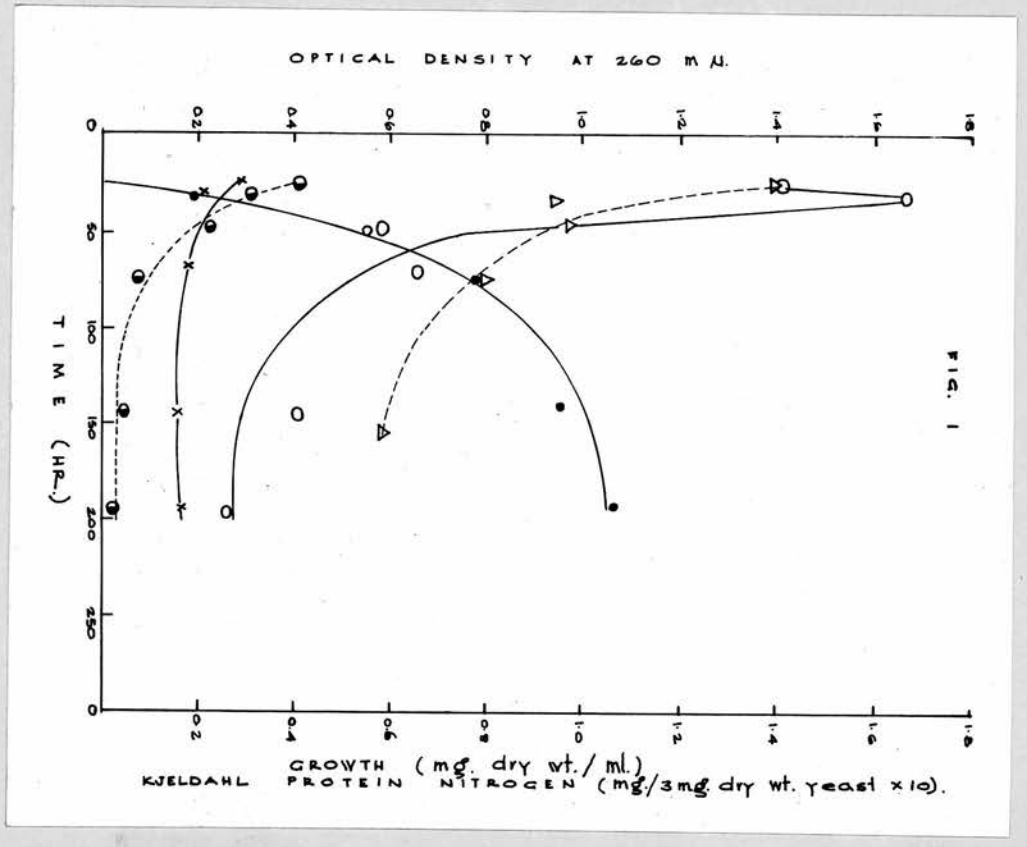

OPTICAL DENSITY AT 260 mμ.
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
50
100
150
200
250
TIME (HR.)
GROWTH (mg. dry wt./ml.)
KJELDAHL PROTEIN NITROGEN (mg./3 mg. dry wt. yeast x 10).

FIG. 1

TABLE I

Effect of incubation time on the size of the intracellular amino acid pool in yeast grown in media containing either an optimal or a suboptimal concentration of biotin

Biotin-optimal		Biotin-suboptimal	
Age (hr.)	Ug. NH$_2$ group per 10.0 mg. dry wt. of yeast	Age (hr.)	Ug. NH$_2$ group per 10.0 mg. dry wt. of yeast
24	108.5	71	92.0
27.5	91.1	96.5	71.0
31	76.0	119	82.0
35	67.2	143.5	81.0
47.5	71.2	167	83.0
73	73.7	239	83.4
122	65.4		

Estimations were carried out on extracts (10.0 ml.) from 10.0 mg. dry weight portions of yeast as described under METHODS.

restriction in growth and change in colour was accompanied by marked changes in the concentrations of DNA, RNA, acid-soluble ultraviolet-absorbing substances and protein. After an initial slight decrease, the concentrations of DNA in both types of yeast remained constant throughout the periods of growth, although under conditions of biotin deficiency the concentration was slightly lower than in the yeast grown in medium containing an optimal concentration of biotin. The sequence of changes observed in the RNA content of the biotin-optimal yeast was similar to that reported by other workers (Di Carlo & Schultz, 1948). In biotin-deficient yeast, however, the concentration of RNA was, by comparison, low and remained so during the observed period of growth. The concentration of Kjeldahl protein nitrogen in the biotin-optimal yeast was highest during the very early stages of the exponential phase of growth, but declined steadily as the culture aged. In the biotin-deficient yeast, the protein nitrogen content increased up to 160 hr., when it was approximately half of that in exponential phase biotin-optimal yeast, but, thereafter it gradually declined. Biotin-optimal yeast contained an appreciable amount of acid-soluble ultraviolet-absorbing substances during the early stages of the exponential phase of growth but, on further incubation, the concentration declined rapidly and, at the end of the exponential phase, had become extremely small. Biotin-deficient yeast

contained significantly higher concentrations of these substances during the early stages of growth although, after about 200 hr., this concentration too had decreased to a low value.

A study of the intracellular amino acid pools of biotin-optimal and biotin-deficient yeast revealed that biotin deficiency had little effect on the concentrations of these pools. Under biotin-optimal conditions this pool decreased as the culture aged, but under biotin deficiency the size of the pool was fairly constant and somewhat higher than in biotin-optimal yeast. However, the amino acid pool under biotin deficiency during the early stages of growth was smaller than that in biotin-optimal yeast.

b) Choice of solvent for the extraction of acid-soluble ultraviolet-absorbing substances and the concentration of these substances in the yeast: The comparatively high concentration of acid-soluble ultraviolet-absorbing substances and the low concentration of RNA and DNA was obviously of interest in relation to the ability of the yeast to synthesize normal amounts of RNA and DNA under conditions of biotin deficiency. It was possible that these ultraviolet-absorbing substances arose as a result of the breakdown of RNA in the biotin-deficient yeast

during extraction with perchloric acid; alternatively, they may have represented purine- and pyrimidine-containing substances that had failed to be polymerised into DNA and RNA. A study was therefore made of the effect of using various acid solvents, including 0.2 N and 1.0 N-perchloric acid, 5% (w/v) and 10% (w/v) trichloroacetic acid and 5% (v/v) n-butanol in M/15 potassium dihydrogen orthophosphate buffer (pH 4.5), to extract these ultraviolet-absorbing substances from 120 hr. biotin-deficient yeast and from exponential phase (40 hr.) and stationary phase (120 hr.) biotin-optimal yeast. Mild extractants, such as water and n-butanol in M/15 potassium dihydrogen orthophosphate buffer (pH 4.5) up to a concentration of 4% (v/v), were found to be totally ineffective in extracting acid-soluble ultraviolet-absorbing substances (Tables II & III). Triplicate 3.0 mg. dry weight portions of washed yeast were taken, and were extracted five or, when necessary, more times with 4.0 ml. portions of the extracting solvent at 3° for 5 min. After centrifugation, the supernatant liquid was decanted, adjusted to pH 4.5, and made up to 5.0 ml. with M/15 potassium dihydrogen orthophosphate buffer (pH 4.5). The optical densities of these extracts were then measured at 260 mμ, with a blank of appropriate reagent. Removal of the ultraviolet-absorbing substances was usually complete in five extractions, although complete removal from biotin-deficient yeast with 5% aqueous butanol required seven separate extractions. After all the

TABLES II and III

Extraction of acid-soluble ultraviolet-absorbing substances from biotin-optimal (40 & 120 hr.) and biotin-deficient (120 hr.) yeast with water (Table II) and 4% (v/v) n-butanol in M/15 phosphate buffer (pH 4.5) (Table III)

Table II

Extraction No.	Optical density at 260 mμ.		
	Biotin-optimal 40 hr.	Biotin-optimal 120 hr.	Biotin-deficient 120 hr.
1.	0.00	0.00	0.00
2.	0.00	0.00	0.00
3.	0.00	0.00	0.00
4.	0.00	0.00	0.00
5.	0.00	0.00	0.00
RNA	0.62	0.193	0.39

Table III

Extraction No.	Optical density at 260 mμ.		
	Biotin-optimal 40 hr.	Biotin-optimal 120 hr.	Biotin-deficient 120 hr.
1.	0.00	0.00	0.00
2.	0.00	0.00	0.00
3.	0.00	0.00	0.001
4.	0.003	0.00	0.007
5.	0.005	0.00	0.01
RNA	0.715	0.372	0.35

Extracts were made on 3.0 mg. dry wt. portions of yeast with separate 4.0 ml. portions of water, 4% (v/v) n-butanol in M/15 phosphate buffer (pH 4.5). Extracts were made up to 5.0 ml. with the extractant used, and the optical densities of these extracts were then measured at 260 mμ. RNA was extracted as described under METHODS.

ultraviolet-absorbing substances had been extracted, the residue was defatted and the RNA extracted and estimated as mentioned under METHODS.

Perchloric acid (0.2 N) extracted all the soluble ultraviolet-absorbing substances fairly rapidly from biotin-deficient yeast and from biotin-optimal yeast. Consistently larger amounts of ultraviolet-absorbing substances were extracted from biotin-deficient yeast than from either exponentially growing or stationary phase yeast grown in presence of an optimal concentration of biotin (Fig. 3). Higher concentrations of perchloric acid (e.g. N) are known to hydrolyse RNA, and this has been used as a means of extracting RNA from tissues (Ogur & Rosen, 1950). However, it would seem from the data shown in Fig. 4 that the RNA in the biotin-deficient yeast was hydrolysed more quickly by N-perchloric acid as compared with RNA in biotin-optimal yeast.

Many workers have recommended the use of trichloroacetic acid for the extraction of ultraviolet-absorbing substances before estimation of nucleic acids in tissues (Davidson, Frazer & Hutchison, 1951; Schmidt & Thannhauser, 1945). Trichloroacetic acid at concentrations 5% (w/v) or 10% (w/v) rapidly extracted the bulk of the acid-soluble ultraviolet-absorbing substances from the biotin-deficient yeast and from the biotin-optimal yeast. The amounts extracted from biotin-deficient yeast again exceeded those

from biotin-optimal yeast (Fig. 5). Little difference was observed between the amounts extracted by 5% and 10% (w/v) trichloroacetic acid, although a slightly decreased content of RNA in biotin-deficient yeast that had been extracted with 10% trichloroacetic acid suggested that some of the nucleic acid may have been hydrolysed during extraction.

Extraction of ultraviolet-absorbing substances by aqueous n-butanol was used by Mitchell and Moyle (1951) in their studies on the chemical anatomy of Staphylococcus aureus (Micrococcus pyogenes). This reagent is far milder than either perchloric acid or trichloroacetic acid, and was used in an attempt to minimize possible hydrolytic breakdown of RNA during extraction. Concentrations of n-butanol up to 4% (v/v) in M/15 potassium dihydrogen orthophosphate buffer (pH 4.5) failed to extract detectable amounts of ultraviolet-absorbing substances from the yeast. But, by using a concentration of 5% (v/v) n-butanol in phosphate buffer, the ultraviolet-absorbing substances were extracted, although at least seven separate extractions were required for complete removal of these substances from biotin-deficient yeast (Fig. 6).

Figures 3, 4, 5 & 6

Extraction of acid-soluble ultraviolet-absorbing substances at 3° from 3.0 mg. dry weight portions of biotin-optimal (40 and 120 hr.) and biotin-deficient (120 hr.) yeast with separate 4.0 ml. portions of 0.2 N perchloric acid (Fig. 3), 1.0 N perchloric acid (Fig. 4), 5% (w/v) trichloroacetic acid (Fig. 5) or 5% (v/v) n-butanol in M/15 potassium dihydrogen orthophosphate buffer (pH 4.5) (Fig. 6). Extracts were adjusted to pH 4.5, made up to 5.0 ml. with M/15 KH_2PO_4, and the optical densities measured at 260 mμ. RNA was extracted as described under METHODS.

FIG. 3

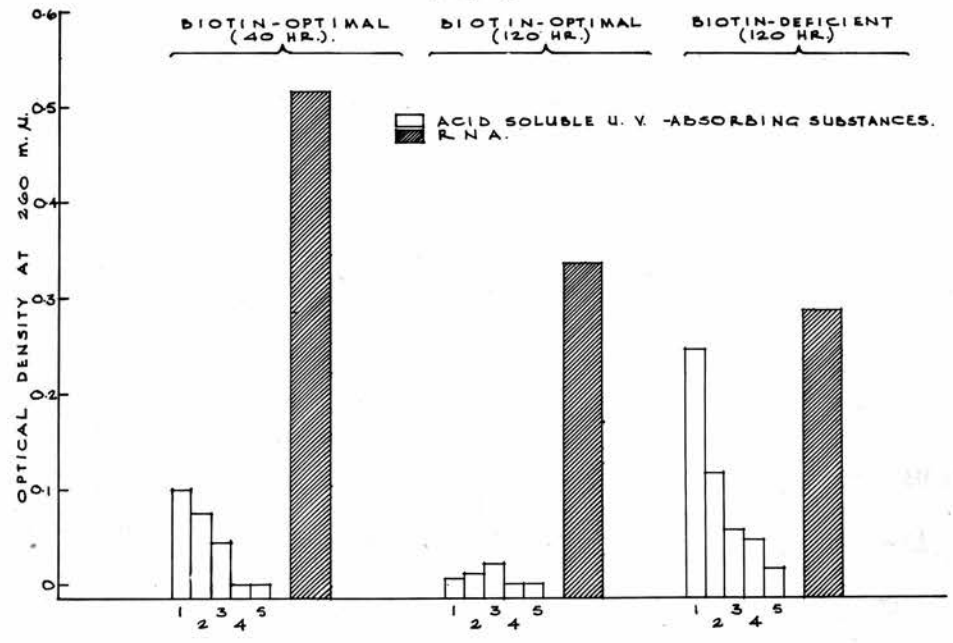

BIOTIN-OPTIMAL (40 HR.).
BIOTIN-OPTIMAL (120 HR.)
BIOTIN-DEFICIENT (120 HR.)
ACID SOLUBLE U. V. -ABSORBING SUBSTANCES.
R N A.
OPTICAL DENSITY AT 260 mμ.
0
0.1
0.2
0.3
0.4
0.5
0.6
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
NUMBER OF EXTRACTIONS.

FIG. 4

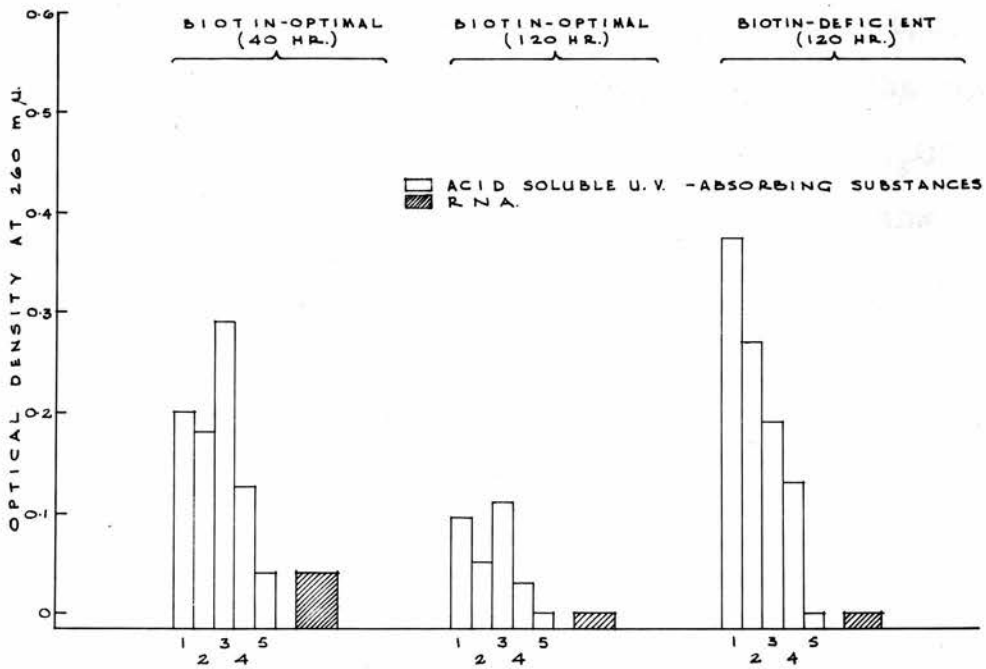

BIOTIN-OPTIMAL (40 HR.)
BIOTIN-OPTIMAL (120 HR.)
BIOTIN-DEFICIENT (120 HR.)
ACID SOLUBLE U. V. -ABSORBING SUBSTANCES
R N A.
OPTICAL DENSITY AT 260 mμ.
0
0.1
0.2
0.3
0.4
0.5
0.6
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
NUMBER OF EXTRACTIONS.

FIG. 5

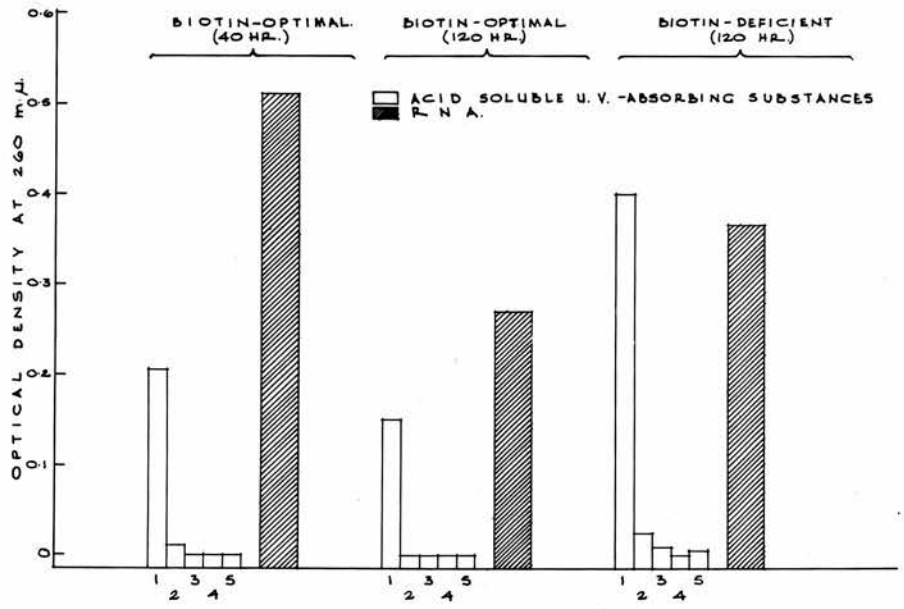
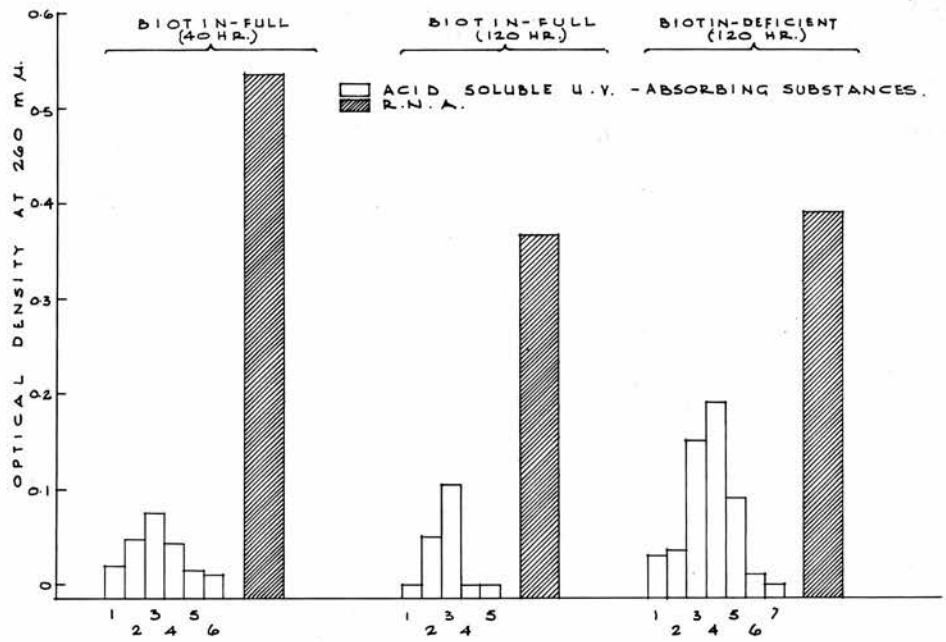

BIOTIN-OPTIMAL (40 HR.)
BIOTIN-OPTIMAL (120 HR.)
BIOTIN-DEFICIENT (120 HR.)
ACID SOLUBLE U.V.-ABSORBING SUBSTANCES
R N A.
OPTICAL DENSITY AT 260 mμ.
0·6
0·5
0·4
0·3
0·2
0·1
0
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
NUMBER OF EXTRACTIONS.

FIG. 6.


BIOTIN-FULL (40 HR.)
BIOTIN-FULL (120 HR.)
BIOTIN-DEFICIENT (120 HR.)
ACID SOLUBLE U.V. -ABSORBING SUBSTANCES.
R.N.A.
OPTICAL DENSITY AT 260 mμ.
0·6
0·5
0·4
0·3
0·2
0·1
0
1 2 3 4 5 6
1 2 3 4 5
1 2 3 4 5 6 7
NUMBER OF EXTRACTIONS.

c) Effect of temperature on the extraction of acid-soluble ultraviolet-absorbing substances from the yeast: Further information about the instability of RNA in biotin-deficient yeast during extraction of the acid-soluble ultraviolet-absorbing substances was obtained when these extractions were carried out at 21° or 30° instead of at 3°. The results obtained showed that elevation of the temperature had no significant effect on the amounts of RNA extracted, as compared with the amounts extracted at the lower temperature (Tables IV-VII). Spectrophotometric examination of the various extracts of acid-soluble ultraviolet-absorbing substances revealed that, in all instances, these showed maximum ultraviolet absorption at or very close to 260 mμ. Absorption data for substances extracted by 0.2 N perchloric acid and 5% (w/v) trichloroacetic acid are shown in Figs. 7 and 8.

d) Effect of biotin deficiency on the nucleotide and nucleobase compositions of yeast RNA: The nucleotide and nucleobase compositions were determined on the RNA extracted from 40 mg. dry weight of yeast. The yeast was extracted with 10.0 ml. portions of 5% (w/v) trichloroacetic acid at 3° until the acid-soluble ultraviolet-absorbing substances had been completely removed; this required five to eight separate extractions. The tissue was defatted by extracting

TABLES IV and V

Effect of temperature on extraction of acid-soluble ultra-violet-absorbing substances from biotin-optimal (40 hr.) (Table IV) and biotin-deficient (120 hr.) (Table V) yeast with 0.2 N perchloric acid

TABLE IV

Extraction No.	Optical density at 260 mμ.		
	Temperature 3°	21°	30°
1.	0.095	0.100	0.092
2.	0.075	0.080	0.083
3.	0.020	0.020	0.000
4.	0.000	0.000	0.000
5.	0.000	0.000	0.000
RNA	0.510	0.493	0.489

TABLE V

Extraction No.	Optical density at 260 mμ.		
	Temperature 3°	21°	30°
1.	0.244	0.241	0.250
2.	0.120	0.114	0.136
3.	0.063	0.088	0.090
4.	0.050	0.068	0.060
5.	0.000	0.004	0.003
RNA	0.300	0.295	0.297

Extracts were made on 3.0 mg. dry weight portions of yeast with separate 4.0 ml. portions of 0.2 N-perchloric acid. Extracts were adjusted to pH 4.5 and made up to 5.0 ml. with phosphate buffer (pH 4.5) and the optical densities of these extracts were then measured at 260 mμ. RNA was extracted as described under METHODS.

TABLES VI and VII

Effect of temperature on extraction of acid-soluble ultra-violet-absorbing substances from biotin-optimal (40 hr.) (Table VI) and biotin-deficient (120 hr.) (Table VII) yeast with 5% (w/v) trichloroacetic acid

TABLE VI

Extraction No.	Optical density at 260 mμ.		
	Temperature 3°	21°	30°
1.	0.205	0.210	0.198
2.	0.050	0.002	0.021
3.	0.000	0.000	0.000
4.	0.000	0.000	0.000
5.	0.000	0.000	0.000
RNA	0.520	0.522	0.519

TABLE VII

Extraction No.	Optical density at 260 mμ.		
	Temperature 3°	21°	30°
1.	0.388	0.358	0.328
2.	0.026	0.013	0.005
3.	0.004	0.000	0.000
4.	0.015	0.016	0.000
5.	0.000	0.000	0.000
RNA	0.295	0.286	0.290

Extracts were made on 3.0 mg. dry weight portions of yeast with separate 4.0 ml. aliquots of 5% (w/v) trichloroacetic acid. Extracts were adjusted to pH 4.5 and made up to 5.0 ml. with phosphate buffer (pH 4.5) and the optical densities of these extracts were then measured at 260 mμ. RNA was extracted as described under METHODS.

Figures 7 & 8

Absorption maxima as determined by spectrophotometric examination of the acid-soluble ultraviolet-absorbing substances obtained by extracting yeast with 0.2 N perchloric acid (Fig. 7) and 5% (w/v) trichloroacetic acid (Fig. 8). Extracts were prepared from 3.0 mg. dry weight portions of biotin-optimal (● ——— ●, 40 hr.) and biotin-deficient (× ——— ×, 120 hr.) yeast with separate 4.0 ml. portions of the extractant. Extracts were adjusted to pH 4.5, made up to 5.0 ml. with M/15 potassium dihydrogen orthophosphate and then the absorption maxima of these extracts were determined against appropriate blanks.

FIG 7

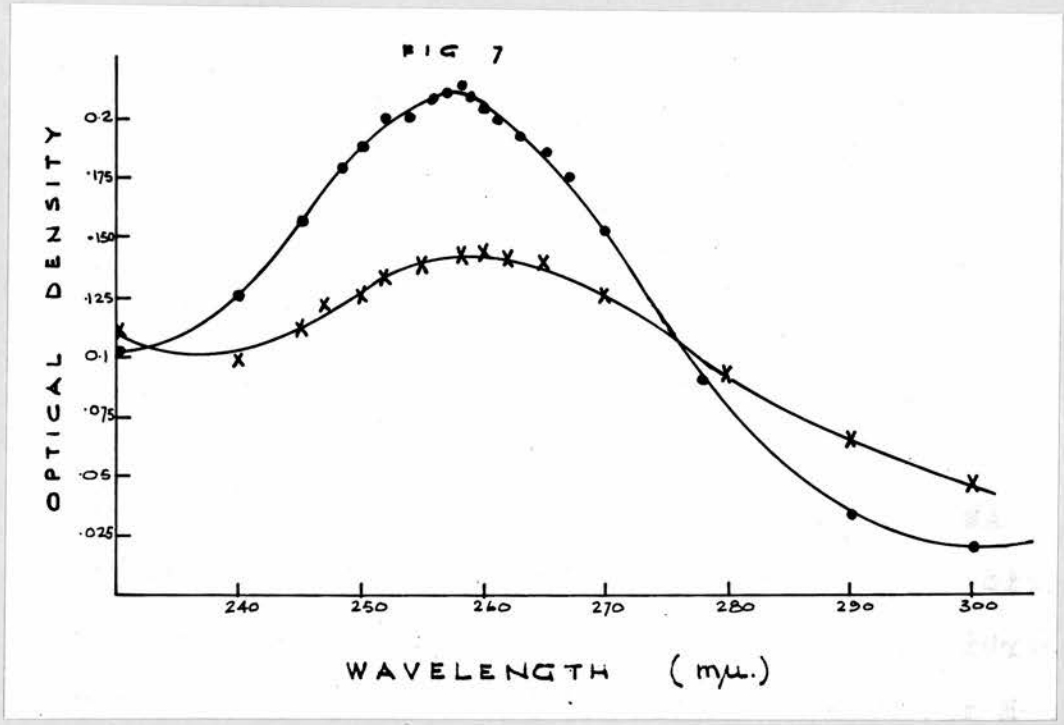
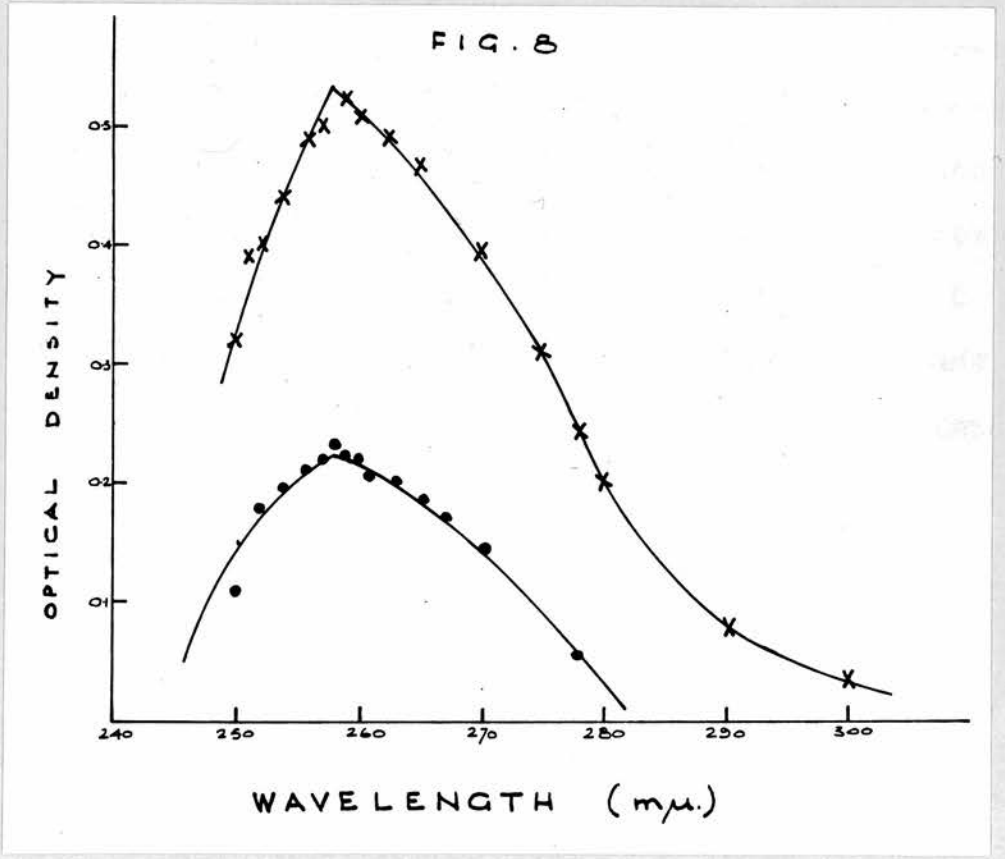

OPTICAL DENSITY
0·2
·175
·150
·125
0·1
·075
·05
·025
240
250
260
270
280
290
300
WAVELENGTH (mμ.)

FIG. 8


OPTICAL DENSITY
0·5
0·4
0·3
0·2
0·1
240
250
260
270
280
290
300
WAVELENGTH (mμ.)

twice with 10.0 ml. portions of a boiling mixture of 95% (v/v) ethanol in water (3 vol.) + ether (1 vol.), and the residue treated with 2.0 ml. of 0.3 N potassium hydroxide for 18 hr. at 37° (Davidson and Smellie, 1952) to hydrolyse polyribonucleotides to soluble mononucleotides. Shorter periods of incubation were tried and, although these were sufficient to allow for the hydrolysis of RNA to acid-soluble nucleotides as detected spectrophotometrically, nevertheless it was shown electrophoretically that incubation for 18 hr. at 37° was necessary to obtain complete hydrolysis to mononucleotides. The nucleotide and nucleobase compositions of the RNA extract were then determined as described under METHODS.

The data in Table VIII show the molar concentrations (with adenylic acid expressed as 10.0) of RNA nucleotides in a sample of commercial RNA and in the RNA from Saccharomyces cerevisiae grown in media containing either an optimal or a suboptimal concentration of biotin. These results show that the molar ratio of purine to pyrimidine nucleotides ranged from 1.03 to 1.13 in the commercial yeast RNA and in biotin-optimal yeast during the exponential and stationary phases of growth. In biotin-deficient yeast from 120 hr. cultures, the ratio was slightly but consistently higher, the average value obtained around 1.28; but in yeast from 7-day biotin-deficient cultures, the ratio had decreased to within the range 1.00-1.15. Closely similar results were

TABLE VIII

Molar concentrations of adenylic, guanylic, cytidylic and uridylic acids (based on adenylic acid = 10.0) in a commercial yeast RNA and in extracts of RNA from Saccharomyces cerevisiae grown in media containing an optimal or a suboptimal concentration of biotin

Source	Age (hr.)	Molar concentrations				Ratio Pu./Py.
		Ad. A.	Gu. A.	Cy. A.	Ur. A.	
Commercial yeast RNA	-	10.0	12.64	7.73	12.2	1.13
		10.0	12.20	7.20	12.62	1.12
		10.0	12.66	7.74	12.61	1.11
Biotin-optimal yeast	40	10.0	11.12	8.00	11.31	1.11
		10.0	10.80	8.10	10.80	1.10
	96	10.0	10.60	8.20	11.60	1.03
Biotin-deficient yeast	120	10.0	11.90	7.4	9.76	1.28
		10.0	11.71	7.28	9.60	1.28
	168	10.0	12.70	8.19	12.02	1.12
		10.0	12.22	7.90	11.02	1.18

Abbreviations used:

Ad. A. - adenylic acid; Gu. A. - guanylic acid;

Cy. A. - cytidylic acid; Ur. A. - uridylic acid;

Pu. - purine and Py. - pyrimidine.

TABLE IX

Molar concentrations of adenine, guanine, cytosine and uracil (based on adenine = 10.0) in a commercial yeast RNA and in extracts of RNA from Saccharomyces cerevisiae grown in media containing an optimal or a suboptimal concentration of biotin

Source	Age (hr.)	Molar concentrations				Ratio Pu./Py.
		Ad.	Gu.	Cy.	Ur.	
Commercial yeast RNA	-	10.0	10.10	8.07	11.03	1.05
		10.0	10.17	8.04	10.90	1.06
Biotin-optimal yeast	40	10.0	11.36	7.91	11.50	1.10
		10.0	11.37	8.00	11.28	1.11
	96	10.0	11.14	8.50	11.40	1.04
		10.0	11.20	8.61	11.37	1.06
Biotin-deficient yeast	120	10.0	11.60	7.56	10.10	1.23
		10.0	11.94	7.37	10.36	1.24
	168	10.0	11.60	9.10	11.90	1.02
		10.0	12.80	8.17	11.50	1.16

Abbreviations used:

Ad. - adenine; Gu. - guanine; Cy. - cytosine;

Ur. - uracil; Pu. - purine and Py. - pyrimidine.

obtained when the ratio of purine to pyrimidine bases in the yeast RNA was determined. As shown in Table IX this ratio was in the range 1.02-1.16 in all the samples of RNA studied, with the exception of that obtained from 120 hr. cultures of biotin-deficient yeast in which it averaged 1.23.

Thanks are due to Dr. N.K. Garg, who did some of the preliminary investigations into the effect of biotin deficiency on the synthesis of nucleic acids and protein by the yeast. However, all of the data presented in this section were obtained independently by the author.

CONCLUSIONS

1. Growth of the yeast under conditions of biotin deficiency was adversely affected; the yeast did not show the sharply defined phases of growth which are characteristic of the yeast grown in biotin-optimal medium.
2. The decreased growth was accompanied by a marked impairment in the ability of the yeast to synthesize nucleic acids and protein. However, in the early stages of growth higher concentrations of acid-soluble ultraviolet-absorbing substances were detected in yeast grown under conditions of

biotin deficiency as compared with yeast grown in biotin-optimal medium.

3. Using different extractants and varying temperatures for the extraction of acid-soluble ultraviolet-absorbing substances, it was concluded that these substances did not arise as a result of the acid degradation of RNA, but were present in the yeast as such. Since these substances absorbed maximally at or very near to 260 mμ, they were taken to be purine- and pyrimidine-containing compounds.

4. Nucleotide and nucleobase analyses, of RNA obtained from yeast grown under different conditions, showed these to have a ratio purine:pyrimidine 1.00-1.15, with the exception of RNA from 5-day cultures of biotin-deficient yeast which had a slightly but consistently higher ratio.

5. Comparison of the intracellular amino acid pools in biotin-optimal and biotin-deficient yeast revealed that, under the conditions of biotin deficiency, the pool during the early stages of growth was smaller than that in biotin-optimal yeast. Later, the size of the pool in biotin-optimal yeast decreased steadily as the culture aged, but the size of the pool remained fairly constant in biotin-deficient yeast.

SECTION II

EFFECT OF VARIOUS BIOTIN-SPARING SUBSTANCES ON GROWTH AND ON SYNTHESIS OF NUCLEIC ACIDS AND PROTEIN BY BIOTIN-DEFICIENT SACCHAROMYCES CEREVISIAE

The results given in the previous section showed that biotin deficiency in Saccharomyces cerevisiae resulted in a decreased synthesis of nucleic acids and protein. The data reported in this section are the results of experiments on the effect of various biotin-sparing substances, including amino acids, purines and related compounds, and fatty acids on growth and on synthesis of nucleic acids and protein by biotin-deficient Saccharomyces cerevisiae. These experiments were carried out in order to evaluate the relative importance of these substances in the metabolism of Saccharomyces cerevisiae.

Survey of the biotin-sparing activity of various substances: Initially a survey was made of the biotin-sparing activity of a number of substances, including amino acids, purines and related compounds, and fatty acids, the biosynthesis of which are known to be biotin-dependent.

The growth-promoting action of each of these substances, with the exception of oleic acid, was found to be complete after about 120 hr. incubation; analyses of nucleic acids, protein, total intracellular amino acid pool and other cell constituents were therefore made on yeast from 120 hr. cultures. An examination was made of the ability of each substance to spare the growth-promoting action of biotin for Saccharomyces cerevisiae and also to restore synthesis of nucleic acids and protein under conditions of relative biotin deficiency.

a) Amino acids: Aspartic acid was the first compound reported to be capable of exerting this sparing action when Koser, Wright and Dorfman (1942) showed that this amino acid was able partially to obviate the biotin requirement of Torula cremoris (Candida pseudotropicalis). Aspartic acid and, to a lesser extent other amino acids, have since been reported to spare the biotin requirement of other micro-organisms including S. cerevisiae (Moat & Emmons, 1954). The effect of L-aspartic acid concentration on growth and on synthesis of nucleic acids and protein by S. cerevisiae in biotin-deficient medium is shown in Fig. 9. There was only a slight stimulation of growth in media supplemented with aspartic acid (Rose,

Figures 9, 10 & 11

Effect of L-aspartic acid concentration (Fig. 9), DL-aspartic acid concentration (Fig. 10) and casamino acids concentration (Fig. 11) on growth (● ——— ●, mg. dry wt./ml. of, and contents of DNA (× ——— ×), RNA (○ ——— ○), acid-soluble ultraviolet-absorbing substances (◒ ----- ◒), Kjeldahl protein nitrogen (△ ----- △, µg./mg. dry wt. yeast), intracellular amino acids (▲ ——— ▲, µg. NH_2/10 mg. dry wt. yeast) and acid-labile phosphate (⊕ ——— ⊕ µmoles P./g. dry wt. of yeast) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin. Yeast was harvested from cultures after 120 hr. incubation at 25°. Contents of DNA, RNA and acid-soluble ultraviolet-absorbing substances are expressed as the optical densities at 260 mµ of extracts from 3.0 mg. dry wt. of yeast made up to 3.0, 10.0 and 5.0 ml. respectively with appropriate extractant.

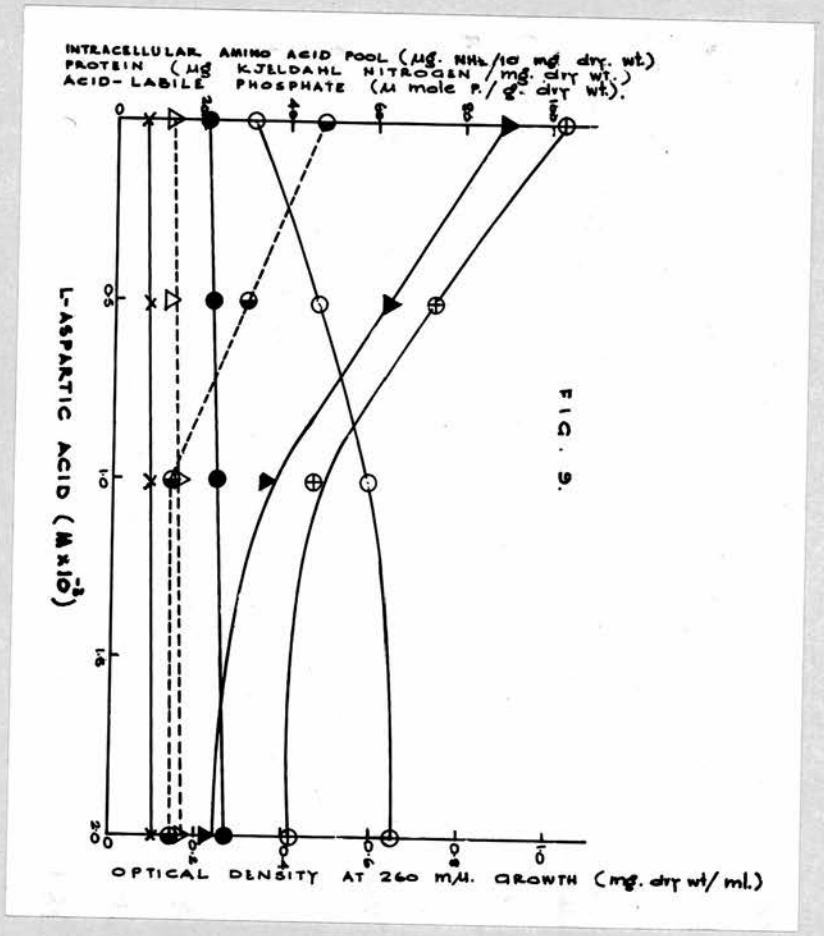

INTRACELLULAR AMINO ACID POOL (μg. NH₂/10 mg dry. wt.)
PROTEIN (μg KJELDAHL NITROGEN / mg. dry wt.)
ACID-LABILE PHOSPHATE (μ mole P./g. dry wt.).
L-ASPARTIC ACID (M x 10⁻³)
OPTICAL DENSITY AT 260 mμ. GROWTH (mg. dry wt/ml.)

FIG. 6.

FIG. 10

OPTICAL DENSITY AT 260 mμ. GROWTH (mg. dry wt. /ml.)
0·0
0·2
0·4
0·6
0·8
1·0
1·0
2·0
3·0
4·0
-3
20
40
60
80
100
DL-ASPARTIC ACID (M x 10⁻³)
KJELDAHL PROTEIN NITROGEN (μg. /mg. dry wt.)
INTRACELLULAR AMINO ACIDS (μg. NH₂/100 mg dry wt.)
ACID-LABILE PHOSPHATE (μ moles P./g. dry wt.)

FIG. 11

INTRACELLULAR AMINO ACID POOL (μg. NH2/10 mg. dry wt. yeast.)
PROTEIN (μg. KJELDAHL NITROGEN /mg. dry wt. yeast.)
ACID-LABILE PHOSPHATE (μ mole P/g. dry wt. yeast.)
OPTICAL DENSITY AT 260 mμ. GROWTH (mg. dry wt./ml.)
CASAMINO ACIDS (mg./ml.)
0
0.5
1.0
1.5
2.0
20
40
60
80
100
2
4
6
8
10

1960, b). Nevertheless, the increase in RNA content of yeast grown in biotin-deficient media containing more than 1.0×10^{-3} M L-aspartic acid or 2.0×10^{-3} M DL-aspartic acid was appreciable, and there was also a rise in the protein content; the DNA content of the yeast also increased. Double the amount of DL-aspartic acid (Fig. 10) was required to produce the same effect as L-aspartic acid, an indication that only the L-isomer was active and also that the D-isomer was not inhibitory. This increased synthesis of high molecular weight cell constituents was accompanied by a depletion of the intracellular pools of amino acids, acid-soluble ultraviolet-absorbing substances and acid-labile phosphate. Yeast grown in biotin-deficient medium containing more than 1.0×10^{-3} M L-aspartic acid or 2.0×10^{-3} M DL-aspartic acid was coloured creamy white instead of the pink colour that is characteristic of biotin-deficient yeast (Chamberlain, Cutts & Rainbow, 1952) and also grew in the form of large aggregates of cells (Dunwell, Ahmad & Rose, 1961).

When biotin-deficient medium was supplemented with casamino acids up to 2.0 mg./ml., there was a greater stimulation of growth than in media supplemented with only aspartate (Fig. 11). The effect of this mixture of amino acids on the DNA and RNA contents of the yeast was similar to that observed in biotin-deficient media supplemented with aspartic acid alone; there was, however, a somewhat

greater increase in the protein content of yeast grown in biotin-deficient media supplemented with casamino acids. The depletion of the pools of amino acids and acid-labile phosphate was less marked than in media supplemented with aspartic acid alone. The greater stimulation of growth in media supplemented with casamino acids suggests the requirement of biotin in the synthesis of other amino acids in addition to aspartic acid. Yeast grown in media containing more than 1.0 mg./ml. casamino acids was coloured creamy white and grew in aggregates.

b) Purines and related compounds: Chamberlain and Rainbow (1954) first reported that adenine was able to spare partially the growth-promoting action of biotin in Saccharomyces cerevisiae. Under the conditions employed in the present study (low concentration of biotin in the medium and use of a small biotin-deficient inoculum), the growth promoting effect of this purine was very slight (Fig. 12). Yeast grown in biotin-deficient medium containing up to 0.5×10^{-3} M adenine contained slightly increased amounts of RNA and protein as compared with yeast grown in unsupplemented biotin-deficient medium. These changes were accompanied by a fall in the content of acid-soluble ultraviolet-absorbing substances and a rise

in the contents of intracellular amino acids and acid-labile phosphate in the yeast. The DNA content of yeast grown in adenine containing biotin-deficient media remained unchanged. Formation of pink pigment was suppressed in yeast grown in media containing more than 0.25×10^{-3} M adenine (Chamberlain & Rainbow, 1954).

Katsuki (1959, a) observed a decrease in the contents of ATP, DPN and Co A in mycelia of Piricularia oryzae as a result of biotin deficiency, which he related to the direct or indirect involvement of biotin in the synthesis of these nucleotide co-enzymes. The drastic diminution in the growth of Saccharomyces cerevisiae under conditions of biotin deficiency has been observed, and experiments were carried out to discover whether this was accompanied by a decrease in the ATP content of the yeast. The results of this investigation are tabulated in Table X. There was indeed a marked decrease in the content of ATP in biotin-deficient yeast, but this deficiency was fully restored on addition of adenine to the biotin-deficient medium. This observation probably explains the results reported by Rose (1960, b) that excretion of nicotinic acid and nicotinic acid-adeninedinucleotide, the two biosynthetic precursors of pyridine nucleotides which appear in the culture medium during growth of S. cerevisiae under conditions of biotin deficiency, is suppressed on adding adenine to the biotin-deficient medium.

Figures 12 & 13

Effect of adenine concentration (Fig. 12) and adenosine concentration (Fig. 13) on growth (●——●, mg. dry wt./ml.) of, and contents of DNA (×——×), RNA (○——○), acid-soluble ultraviolet-absorbing substances (◒ - - - - ◒), Kjeldahl protein nitrogen (△ - - - - △, µg./mg. dry wt.), intracellular amino acids (▲——▲, µg. NH_2/10.0 mg. dry wt.), and acid-labile phosphate (⊕——⊕, µmoles P./g. dry wt.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin. Yeast was harvested from cultures after 120 hr. at 25°. Contents of DNA, RNA and acid-soluble ultraviolet-absorbing substances are expressed as the optical densities at 260 mµ of extracts from 3.0 mg. dry wt. of yeast made up to 3.0, 10.0 and 5.0 ml. respectively.

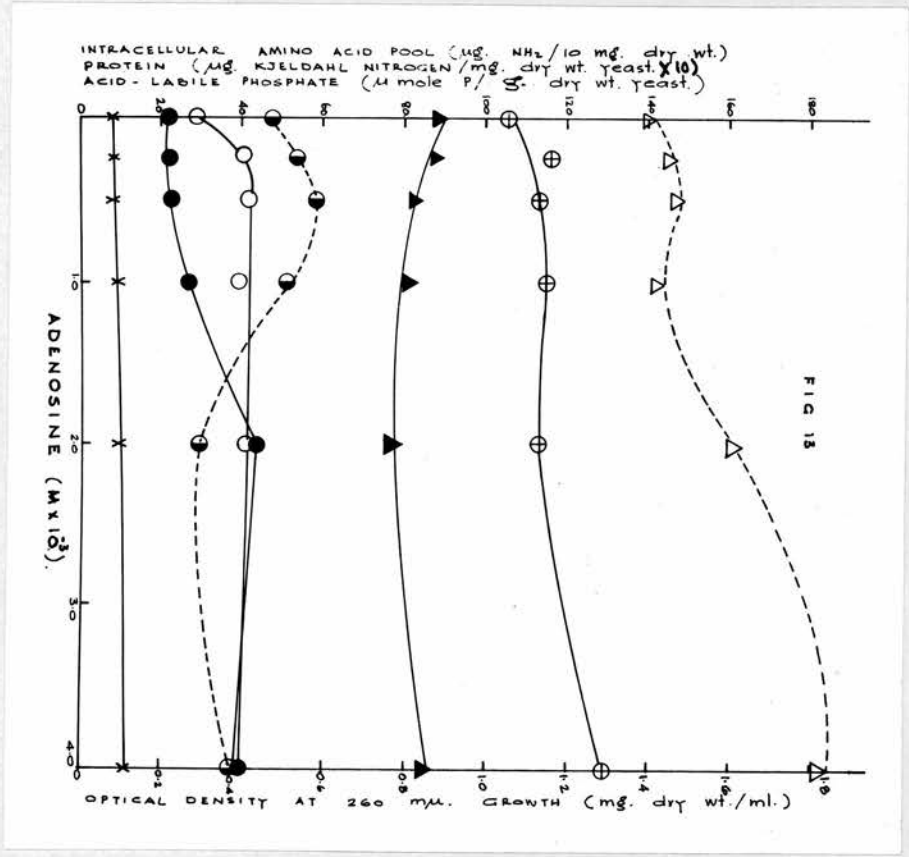

INTRACELLULAR AMINO ACID POOL (µg. NH2/10 mg. dry wt.)
PROTEIN (µg. KJELDAHL NITROGEN/mg. dry wt. yeast. X10)
ACID-LABILE PHOSPHATE (µ mole P/g. dry wt. yeast.)
ADENOSINE (M x 10³).
OPTICAL DENSITY AT 260 mµ.
GROWTH (mg. dry wt./ml.)

FIG 13

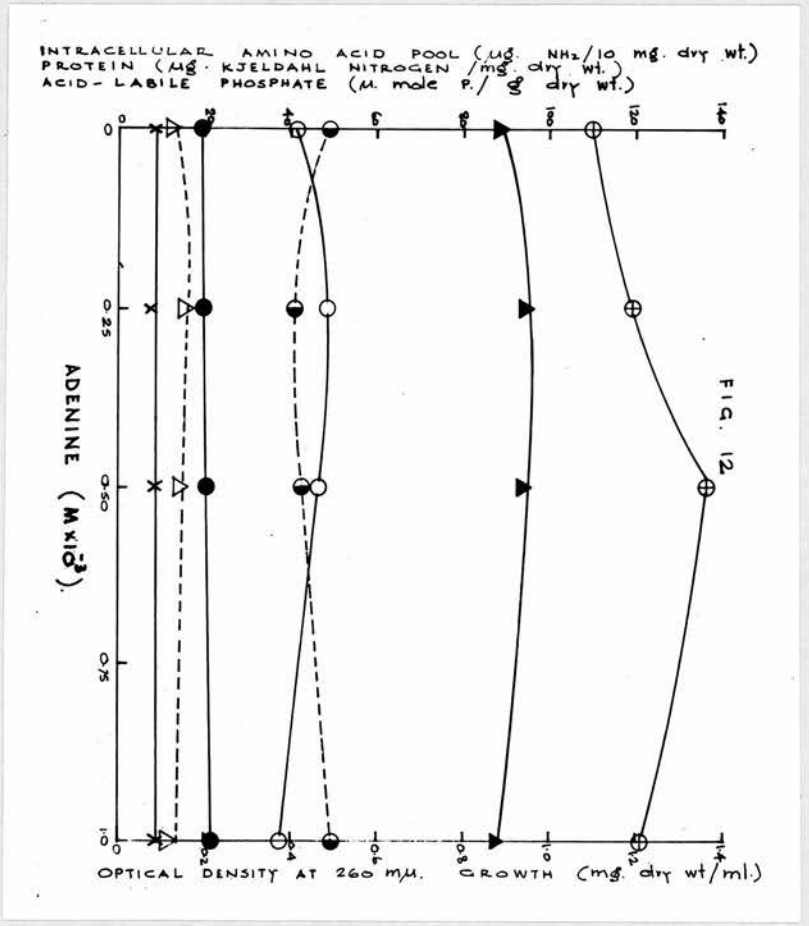

INTRACELLULAR AMINO ACID POOL (µg. NH2/10 mg. dry wt.)
PROTEIN (µg. KJELDAHL NITROGEN/mg. dry wt.)
ACID-LABILE PHOSPHATE (µ. mole P./g. dry wt.)
ADENINE (M x 10³).
OPTICAL DENSITY AT 260 mµ.
GROWTH (mg. dry wt/ml.)

FIG. 12

TABLE X

Adenosine triphosphate content of yeast grown in medium containing an optimal (8.0×10^{-10} M) or a suboptimal (0.4×10^{-10} M) concentration of biotin in the presence and absence of adenine

Yeast	adenine concen. (M x 10^{-3})	Age (hr.)	ATP (µmoles per g. dry wt. of yeast)
Biotin-optimal	-	36	8.1
	-	44	10.0
	-	64	7.2
Biotin-deficient	-	120	0.0 to 1.8
"	0.25	120	4.7
"	0.50	120	7.3
"	1.00	120	11.4

Yeast was harvested by centrifugation and the crop washed thrice with ice-cold water and thrice with ice-cold acetone. Acetone-dried powders were then prepared and were used for the extraction of ATP, which was estimated by dephosphorylation with myosin B as described under METHODS.

The purine guanine (Table XI) and the pyrimidines cytosine, uracil and thymine (Table XII) when added together were found not to affect growth or colour of, or the contents of nucleic acids, protein and related substances in, biotin-deficient yeast. In fact, a little inhibition in growth was observed when a mixture of these pyrimidines was included in the biotin-deficient medium. Each of the pyrimidines was incorporated in biotin-deficient medium in concentrations up to 1.0×10^{-3} M; because of its relative insolubility, guanine was tested only in concentrations up to 0.3×10^{-3} M. When a mixture of adenine + guanine (Table XIII) or adenine + guanine + cytosine + uracil + thymine (Table XIV) was included in the biotin-deficient medium, no changes in the yeast composition were observed as compared with yeast grown in media supplemented with only adenine (Fig. 12).

Adenosine was the only nucleoside tested which had any effect on growth of, or content of nucleic acids and protein in, biotin-deficient yeast (Fig. 13). Stimulation of growth was greatest in media containing 2.0×10^{-3} M adenosine. There was an increase in the RNA content in yeast grown in media containing 0.5×10^{-3} M adenosine as compared with the content in yeast grown in unsupplemented biotin-deficient medium, but higher concentrations (4.0×10^{-3} M) were required to bring about an appreciable increase in the protein content. These slight increases in nucleic acid

TABLE XI

Effect of guanine concentration (Gu. Conc.) on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A-S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Gu. Conc. ($M \times 10^{-3}$)	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.)	Amino acids (µg. NH_2/ 10.0 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
0.00	0.194	0.491	0.402	0.093	13.6	96.2	101.1
0.10	0.198	0.584	0.372	0.091	14.8	101.2	111.0
0.20	0.199	0.603	0.345	0.094	14.2	98.4	108.0
0.30	0.200	0.641	0.398	0.089	14.63	102.6	111.0

Yeast was harvested from cultures after 120 hr. at 25°. Contents of acid-soluble ultraviolet-absorbing substances, RNA and DNA are expressed as the optical densities at 260 mµ of the extracts obtained from 3.0 mg. dry wt. portions of yeast and made up to 5.0, 10.0 and 3.0 ml. respectively.

TABLE XII

Effect of the concentration of a mixture of cytosine, uracil and thymine (Cy., Ur. & Thy.) on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A.S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Conc. of Cy., Ur. & Thy. each in ($M \times 10^{-3}$)	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.)	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
0.00	0.224	0.459	0.424	0.087	16.2	90.0	117.0
0.25	0.210	0.537	0.393	0.089	18.1	94.8	106.0
0.50	0.195	0.505	0.401	0.086	14.8	91.6	120.0
1.00	0.173	0.488	0.382	0.084	12.1	92.3	117.0

Yeast was harvested from cultures after 120 hr. at 25°. Contents of acid-soluble ultraviolet-absorbing substances, RNA and DNA are expressed as the optical densities at 260 mµ of the extracts obtained from 3.0 mg. dry weight portions of yeast and made up to 5.0, 10.0 and 3.0 ml. respectively.

TABLE XIII

Effect of the concentration of a mixture of adenine (Ad.) and guanine (Gu.) on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A-S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj.-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Conc. of Ad. ($M \times 10^{-3}$)	Conc. of Gu. ($M \times 10^{-3}$)	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
0.00	0.00	0.213	0.554	0.301	0.087	15.2	88.1	114.0
0.25	0.05	0.215	0.474	0.356	0.097	18.3	90.3	136.0
0.50	0.10	0.220	0.508	0.380	0.090	15.0	82.6	141.0
1.00	0.20	0.221	0.610	0.359	0.092	16.6	86.8	135.0

Yeast was harvested from cultures after 120 hr. at 25°. Contents of acid-soluble ultraviolet-absorbing substances, RNA and DNA are expressed as the optical densities at 260 mµ of the extracts obtained from 3.0 mg. dry weight portions of yeast and made up to 5.0, 10.0 and 3.0 ml. respectively.

TABLE XIV

Effect of the concentration of a mixture of adenine (Ad.), guanine (Gu.), cytosine (Cy.), uracil (Ur.) and thymine (Thy.) on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A-S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Conc. of Ad., Cy., Ur. & Thy. each in ($M \times 10^{-3}$)	Conc. of Gu. in ($M \times 10^{-3}$)	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
0.00	0.00	0.214	0.44	0.384	0.093	16.8	86.3	143.0
0.25	0.05	0.238	0.358	0.426	0.101	17.0	92.0	153.0
0.50	0.10	0.236	0.422	0.441	0.100	16.3	88.6	147.0
1.00	0.20	0.234	0.544	0.390	0.103	15.4	90.0	152.0

Yeast was harvested from cultures after 120 hr. at 25°. Contents of acid-soluble ultraviolet-absorbing substances, RNA and DNA are expressed as the optical densities at 260 mµ of the extracts obtained from 3.0 mg. dry weight portions of yeast and made up to 5.0, 10.0 and 3.0 ml. respectively.

and protein content were accompanied by a small decrease in the amounts of intracellular amino acids and acid-soluble ultraviolet-absorbing substances; however, in the presence of higher concentrations (2.0-4.0 x 10^{-3} M) of adenosine, the amounts of these low molecular weight substances increased. Growth in adenosine-containing biotin-deficient media had no detectable effect on the DNA content of the yeast. Although adenosine was capable of stimulating growth and synthesis of RNA and protein, yeast grown in biotin-deficient media containing up to 4.0 x 10^{-3} M of this nucleoside remained pink in colour.

There was no stimulation of yeast growth in biotin-deficient media supplemented with a mixture of adenine (up to 1.0 x 10^{-3} M) + D-ribose (up to a concentration of 2.0 x 10^{-3} M) or D-ribose alone (Table XV); also, the nucleic acid and protein content of the yeast grown in these media was the same as in yeast grown in unsupplemented biotin-deficient medium, and there was no change in pools of intracellular amino acids and acid-labile phosphate.

The only nucleotide tested for biotin-sparing activity was adenosine-2($\overline{3}$)-phosphate (yeast adenylic acid). It was shown, however, that, when this compound was incorporated in the biotin-deficient medium up to 2.0 x 10^{-3} M, there was no detectable effect on growth or colour of, or on the contents of nucleic acids and protein and related compounds in, the yeast (Table XVI).

TABLE XV

Effect of the concentration of ribose (Rib.) and a mixture of ribose and adenine (Ad.) on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A-S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Rib. Conc. ($M \times 10^{-3}$)	Ad. Conc. ($M \times 10^{-3}$)	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
0.00	0.00	0.199	0.405	0.400	0.092	13.7	100.0	113.0
1.00	0.00	0.196	0.410	0.396	0.095	14.7	96.0	112.0
1.00	1.00	0.209	0.430	0.420	0.107	14.4	100.0	125.0
2.00	2.00	0.209	0.510	0.392	0.094	13.9	97.0	141.0

Yeast was harvested from cultures after 120 hr. at 25°. Contents of acid-soluble ultraviolet-absorbing substances, RNA and DNA are expressed as the optical densities at 260 mµ of the extracts obtained from 3.0 mg. dry weight portions of yeast and made up to 5.0, 10.0 and 3.0 ml. respectively.

TABLE XVI

Effect of adenylic acid (Ad. A.) concentration on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A-S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Ad. A. Conc. ($M \times 10^{-3}$)	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
0.00	0.198	0.451	0.387	0.097	13.9	94.0	103.0
0.50	0.207	0.480	0.354	0.093	15.6	80.0	99.0
1.00	0.205	0.443	0.373	0.094	14.3	96.0	105.0
2.00	0.209	0.435	0.373	0.094	14.4	90.0	111.0

Yeast was harvested from cultures after 120 hr. at 25°. Contents of acid-soluble ultraviolet-absorbing substances, RNA and DNA are expressed as the optical densities at 260 mµ of the extracts obtained from 3.0 mg. dry weight portions of yeast and made up to 5.0, 10.0 and 3.0 ml. respectively.

c) Fatty acids: The ability of oleic acid to spare the growth promoting action of biotin was first reported for Lactobacillus casei by Williams and Fieger (1946). Elaidic acid, which was present in the sample of oleic acid used, has also been shown to possess biotin-sparing action for some micro-organisms (Cheng, Greenberg, Deuel & Melnick, 1951), as also have certain other unsaturated fatty acids having a chain length of 12 or more carbon atoms (Hofmann, O'Leary, Yoho & Liu, 1959).

When oleic acid, in concentration up to 100 µg./ml., was included in biotin-deficient medium, there was a stimulation of yeast growth which continued as cultures were incubated for longer periods and only levelled off after about 250 hr. incubation (Fig. 14). Prolonged incubation was shown to be accompanied by a slight fall in the contents of RNA and protein in the yeast and a depletion of the intracellular acid-soluble ultraviolet-absorbing substances and amino acids. There was, however, a slight increase in the DNA content of yeast grown in biotin-deficient media supplemented with oleic acid; also, the yeast remained pink in colour.

d) Effect of mixtures of biotin-sparing substances: From the results reported in section II a, b and c it was

Figure 14

Effect of incubation time on growth (●——●, mg. dry wt./ml.) of, and contents of DNA (×——×), RNA (○——○), acid-soluble ultraviolet-absorbing substances (◒-----◒), Kjeldahl protein nitrogen (△-----△, μg. K_j-N/mg. dry wt.), and intracellular amino acids (▲——▲, μg. NH_2/10 mg. dry wt.) in, yeast grown in a medium containing a suboptimal (0.4×10^{-10} M) concentration of biotin and supplemented with oleic acid (100 ug. per ml.). Contents of DNA, RNA and acid-soluble ultraviolet-absorbing substances are expressed as the optical densities at 260 mμ of extracts from 3.0 mg. dry wt. of yeast made up to 3.0, 10.0 and 5.0 ml. respectively.

FIG. 14
OPTICAL DENSITY AT 260 MM. GROWTH (mg. dry wt/ml.)
INTRACELLULAR AMINO ACID POOL (μg. NH2/10 mg. dry wt. yeast.)
PROTEIN (μg. KJELDAHL NITROGEN/mg. dry wt. yeast.)
TIME (HOUR.)
0
0.2
0.4
0.6
0.8
1.0
1.2
20
40
60
80
100
120
50
100
150
200
250
300
350

apparent that adenine, adenosine, aspartic and casamino acids were each capable of sparing the growth-promoting action of biotin to some extent and, with the exception of oleic acid, of partially restoring synthesis of nucleic acids and protein. A study was then made of the effect of certain binary, tertiary and quabernary mixtures of these biotin-sparing substances on growth of, and synthesis of nucleic acids and protein by, biotin-deficient yeast in order to discover whether there exist synergistic relationships among the actions of these substances. The results of this study are summarised in Table 17. The growth promoting effect of most of the combinations tested was approximately additive. However, the increase in growth in media supplemented with oleic acid + aspartic acid was appreciably greater than the sum of the increases obtained when each of these compounds was present singly in the medium. This synergistic effect was also observed when a mixture of adenosine + aspartic acid + oleic acid or casamino acids instead of aspartic acid was included in the biotin-deficient medium, although the magnitude of the effect was approximately the same as in medium supplemented with aspartic acid + oleic acid. Yeast grown in biotin-deficient media containing either aspartic acid or casamino acids, in the presence or absence of other biotin-sparing substances, grew in the form of large aggregates of cells (Dunwell, Ahmad & Rose, 1961).

TABLE XVII

Effect of mixtures of biotin-sparing substances on growth of, and contents of acid-soluble ultraviolet-absorbing substances (A-S. u-v. abs. subs.), RNA, DNA, Kjeldahl protein nitrogen (Kj-N), intracellular amino acids and acid-labile phosphate (A-L. Phos.) in, yeast grown in media containing a suboptimal (0.4×10^{-10} M) concentration of biotin

Additions to biotin-deficient medium	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
None	0.21	0.512	0.32	0.09	13.9	88.0	103.0
Ad.	0.22	0.499	0.37	0.08	14.9	88.6	121.15
Adn.	0.27	0.510	0.38	0.09	14.4	84.0	116.0
Asp. A.	0.29	0.136	0.65	0.11	18.9	24.0	41.6
Cas. A.	0.42	0.220	0.56	0.13	21.7	60.0	79.1
Ol. A.	0.34	0.447	0.32	0.12	15.6	82.0	114.0
Ad. + Cas. A.	0.37	0.240	0.65	0.16	22.4	52.2	60.0
Adn. + Cas. A.	0.545	0.242	0.566	0.131	17.0	64.0	-

TABLE XVII (contd.)

Additions to biotin-deficient medium	Growth (mg. dry wt./ml.)	A-S. u-v. abs. subs.	RNA	DNA	Protein (µg. Kj-N/ mg. dry wt.	Amino acids (µg. NH_2/ 10 mg. dry wt.)	A-L. Phos. (µmoles P./ g. dry wt.)
Cas. A. + Ol. A.	0.586	0.269	0.57	0.137	19.8	71.0	68.3
Ad. + Ol. A.	0.360	0.497	0.376	0.080	13.8	82.0	129.4
Adn. + Asp. A.	0.340	0.121	0.733	0.133	23.8	24.0	43.9
Adn. + Ol. A.	0.390	0.370	0.310	0.090	13.0	82.0	103.8
Adn. + Ol. A. + Asp. A.	0.630	0.145	0.500	0.139	15.4	53.0	-
Adn. + Cas. A. + Ol. A.	0.620	0.191	0.580	0.136	21.6	62.0	-
Asp. A. + Cas. A. + Ol. A.	0.553	0.293	0.730	0.166	20.8	58.0	53.5
Ad. + Asp. A. + Cas. A. + Ol. A.	0.585	0.266	0.751	0.125	26.9	46.0	68.3
Adn. + Asp. A. + Cas. A. + Ol. A.	0.618	0.268	0.718	0.133	25.6	51.0	69.4

'-' means not estimated

TABLE XVII (contd.)

Abbreviations used:

Ad. - adenine; Adn. - adenosine; Asp. A. - L-aspartic acid;

Cas. A. - casamino acids; Ol. A. - oleic acid.

Biotin-sparing substances were incorporated in biotin-deficient medium at the following concentrations:

Adenine (1.0×10^{-3} M), Adenosine (1.0×10^{-3} M), L-aspartic acid (2.0×10^{-3} M), Casamino acids (2.0 mg./ml.) and Oleic acid (100 µg./ml.)

Cultures were removed after 120 hr. incubation at 25° and, after growth had been measured, the yeast was harvested by centrifugation, washed and analysed.

e) Growth of the yeast in media containing mixtures of certain biotin-sparing substances: It was apparent from the data presented in Table XVll that growth of, and synthesis of nucleic acids and protein by, biotin-deficient Saccharomyces cerevisiae was restored, to an appreciable extent, by mixtures of certain biotin-sparing substances. However, biotin-deficient yeast differs from that grown under biotin-optimal conditions not only in containing diminished amounts of nucleic acids and protein but also in that, during the growth cycle, it does not show the sequence of changes in nucleic acids and protein content (compare Figs. 1 & 2). Experiments were, therefore, conducted to discover whether the increased synthesis of nucleic acids and protein in yeast grown in media containing mixtures of certain biotin-sparing substances was accompanied by changes in contents of nucleic acids and protein similar to those occurring in biotin-optimal yeast. Data in Fig. 15 show that, in yeast grown in biotin-deficient medium supplemented with aspartic acid + oleic acid, the pattern of changes in the RNA and protein contents was qualitatively similar to that which occurs during growth of biotin-optimal yeast (Fig. 1). Similarly, when adenine and casamino acids were included in the biotin-deficient medium in addition to aspartic acid + oleic acid (Fig. 16), the pattern of changes was identical with that observed in media containing only aspartic and oleic acids. With both of these combinations it was observed that,

Figure 15

Effect of incubation time on growth (●——●, mg. dry wt./ml. x 2) of, and contents of DNA (×——×), RNA (○——○), acid-soluble ultraviolet-absorbing substances (◒-----◒), Kjeldahl protein nitrogen (△----- △ x 2, μg./mg. dry wt.), intracellular amino acids (▲——▲, μg. NH_2/10.0 mg. dry wt.) in, yeast grown in a medium containing a suboptimal (0.4×10^{-10} M) concentration of biotin and supplemented with L-aspartic acid (2.0×10^{-3} M) + oleic acid (100 μg./ml.). Contents of DNA, RNA and acid-soluble ultraviolet-absorbing substances are expressed as the optical densities at 260 mμ. of extracts from 3.0 mg. dry wt. of the yeast made up to 3.0, 10.0 and 5.0 ml. respectively.

FIG. 15
OPTICAL DENSITY AT 260 MM. GROWTH (mg. dry wt./ml x 2)
KJELDAHL PROTEIN NITROGEN (µg./mg. dry wt. %2)
INTRACELLULAR AMINO ACIDS (µg. NH2/10.0 mg. dry wt.)
TIME (Hr.)
0
0.2
0.4
0.6
0.8
1.0
20
40
60
80
100
40
80
120
160
200
240
280

Figure 16

Effect of incubation time on growth (●——●, mg. dry wt./ml. x 2) of, and contents of DNA (×——×), RNA (○——○), acid-soluble ultraviolet-absorbing substances (◒-----◒), Kjeldahl protein nitrogen (△-----△, µg./mg. dry wt.) and intracellular amino acids (▲——▲, µg. NH_2/10 mg. dry wt.) in, yeast grown in a medium containing a suboptimal (0.4×10^{-10} M) concentration of biotin and supplemented with adenine (1.0×10^{-3} M) + L-aspartic acid (2.0×10^{-3} M) + casamino acids (2.0 mg./ml.) + oleic acid (100 µg./ml.). Contents of DNA, RNA and acid-soluble ultraviolet-absorbing substances are expressed as the optical densities at 260 mµ of extracts from 3.0 mg. dry wt. of the yeast made up to 3.0, 10.0 and 5.0 ml. respectively.

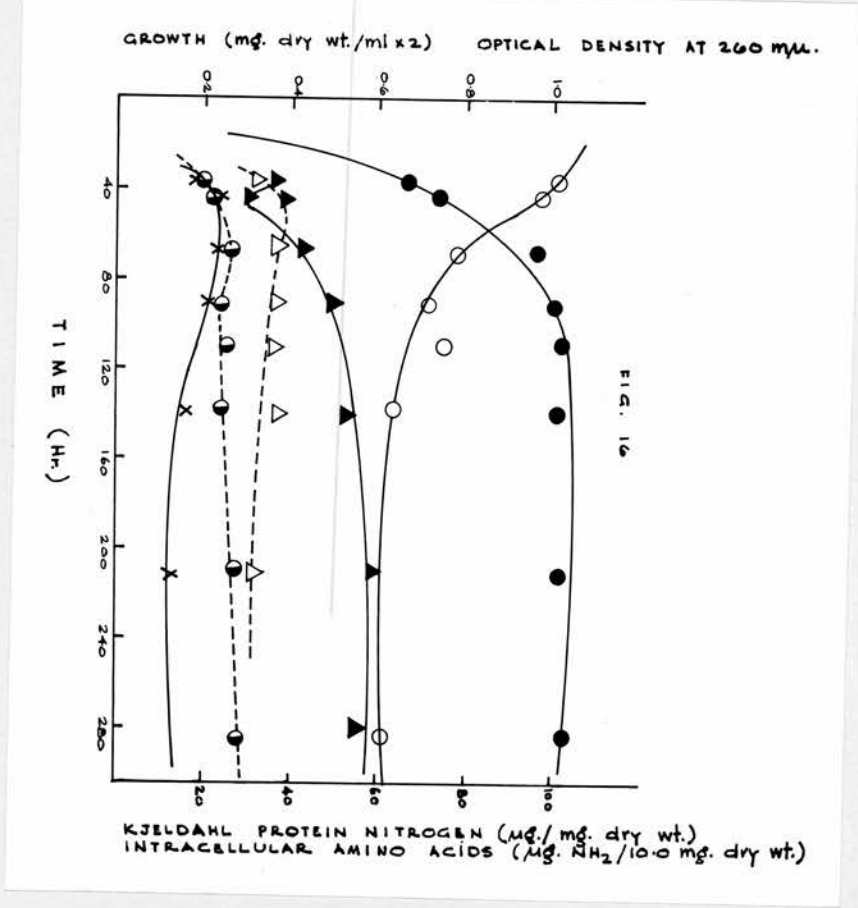

GROWTH (mg. dry wt./ml x 2)
OPTICAL DENSITY AT 260 mμ.
0.2
0.4
0.6
0.8
1.0
40
80
120
160
200
240
280
TIME (Hr.)
20
40
60
80
100
KJELDAHL PROTEIN NITROGEN (μg./ mg. dry wt.)
INTRACELLULAR AMINO ACIDS (μg. NH2/10.0 mg. dry wt.)

FIG. 16

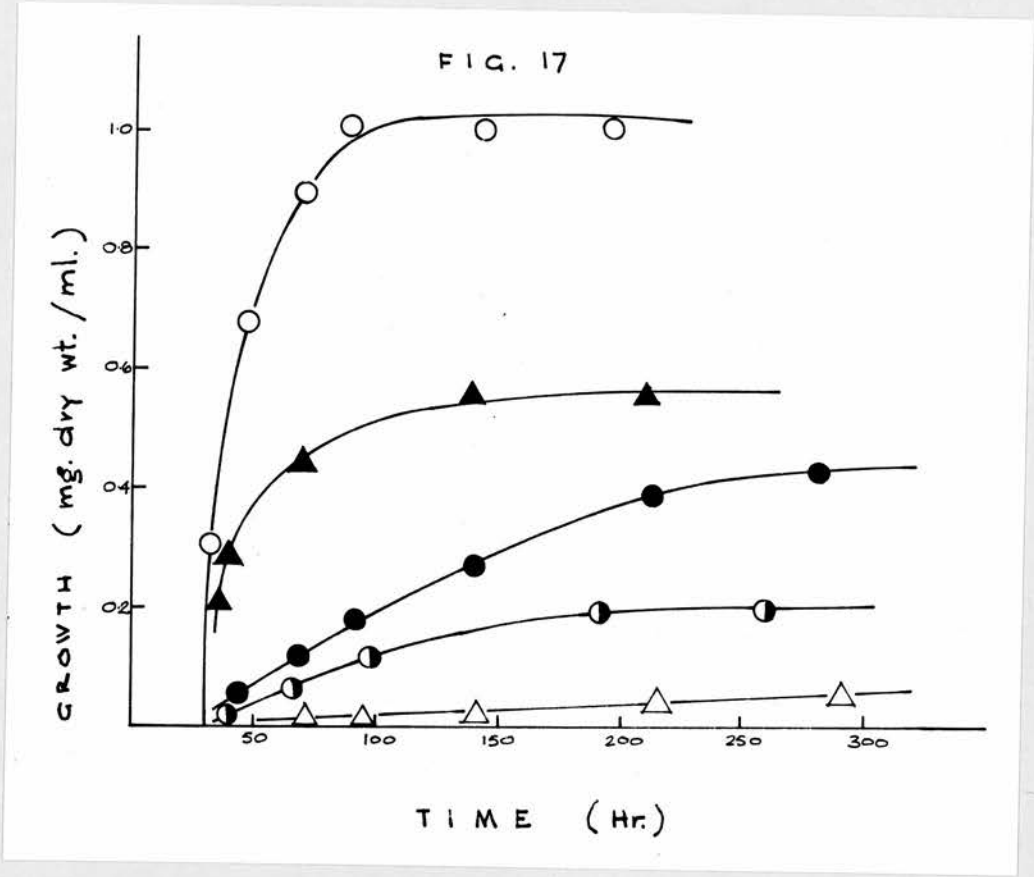


Figure 17

Effect of incubation time on growth of the yeast in media containing an optimal (8.0×10^{-10} M) concentration of biotin (○——○), a suboptimal (0.4×10^{-10} M) concentration of biotin (●——●), and a suboptimal concentration of biotin but supplemented with L-aspartic acid (2.0×10^{-3} M) + oleic acid (100 µg./ml.) (▲——▲). Curves showing growth in biotin-free medium (△——△) and in biotin-free medium supplemented with L-aspartic acid (2.0×10^{-3} M) + oleic acid (100 µg./ml.) (◒——◒) are also shown.

during the early part of the exponential phase of growth, the protein content of the yeast was almost equal to that in early exponential phase biotin-optimal yeast. In these cultures, there was, moreover, a sharply defined exponential phase of growth, followed by a stationary phase; these phases of growth were less well defined in cultures of the yeast grown in unsupplemented biotin-deficient media (Fig. 17).

Although mixtures of certain biotin-sparing substances brought about a very appreciable restoration of nucleic acid and protein synthesis, the cell crop in stationary phase cultures of the yeast grown in these media was still only approximately half of that in stationary phase biotin-optimal cultures. Also, the amount of growth in biotin-free medium containing aspartic acid + oleic acid was still only a small fraction of the amount in stationary phase biotin-optimal cultures (Fig. 17).

CONCLUSIONS

1. Biotin-deficient yeast was found to contain decreased amounts of ATP. However, ATP synthesis was restored in yeast when grown in biotin-deficient medium containing adenine.

2. Adenine, adenosine, L-aspartic acid and casamino acids have each been shown to stimulate growth of, and to bring about partial restoration of nucleic acids and protein synthesis by the yeast under conditions of biotin deficiency. The most marked restoration of nucleic acids and protein synthesis was brought about by aspartic acid or casamino acids.

3. Oleic acid also stimulated growth of biotin-deficient yeast, but this fatty acid was shown to be ineffective in restoring nucleic acid and protein synthesis. It was concluded that the biotin-sparing action of oleic acid was fundamentally different from the actions of other biotin-sparing compounds tested.

4. A synergistic action in the ability of a mixture of aspartic acid and oleic acid to spare the growth promoting action of biotin was observed.

5. During growth of the yeast in biotin-deficient media supplemented with a mixture of aspartic acid + oleic acid or with certain other mixtures of biotin-sparing substances, there was a well defined exponential phase of growth, similar to that observed in yeast grown in medium containing an optimal concentration of biotin.

SECTION III

ACTIVITIES OF CERTAIN ENZYMES IN BIOTIN-DEFICIENT SACCHAROMYCES CEREVISIAE

During the last part of the present study an investigation was undertaken to discover the effects of biotin deficiency on the activities in the yeast of four enzymes, namely, acid-pyrophastase, invertase, malic dehydrogenase and carbamyl phosphate ornithine carbamyl transferase (CPOC transferase). The activities of these four enzymes were taken as parameters for the synthesis of specific proteins. The yeast was grown in biotin-deficient medium supplemented with oleic acid, aspartic acid or a mixture of aspartic acid + oleic acid as well as in biotin-optimal and biotin-deficient media and the activities of each of the four enzymes were determined in yeast grown in each of these media. After harvesting, the yeast crop obtained was washed thrice with ice-cold water and disrupted in the Mickle disintegrator as described under METHODS.

a) Fragility of yeast during disruption in the Mickle disintegrator: Numerous workers have used the Mickle disintegrator for disrupting different micro-organisms and for the preparation of cell-free extracts in the determination of activities of various enzymes. Although a standard time of 30 min. was used and the same conditions were rigidly adhered to, the breakage of yeast grown in various media produced quite different results (Table XVIII). The data obtained showed that, whereas the percentage of the total cell protein obtained in the cell-free extracts of biotin-optimal yeast was between 16 and 36%, in yeast grown in unsupplemented biotin-deficient medium, over 96% of the total cell protein appeared in the extract, showing not only that this yeast was more fragile but that the cell wall contained an extremely small amount of protein. Comparative fragility of the yeast grown under conditions of biotin deficiency is apparent from Fig. 18. In oleic acid-supplemented yeast, over 80% of the total protein appeared in the cell-free extract. However, in yeast grown in biotin-deficient medium supplemented with aspartic acid, in the presence or absence of oleic acid, the recovery of total protein in the cell-free extract never exceeded 22%.

TABLE XVIII

Percentage concentrations of protein in cell-free extracts of yeast grown in various media

Media in which yeast was grown	Age (hr.)	Whole cells Total protein (mg. protein/ 100 mg. dry wt. of yeast)	Cell-free extract (mg. protein/5.0 ml. of extract obtained by disrupting 100 mg. dry wt. of yeast)	Percentage total cell protein in cell-free extract
Biotin-optimal	33	19.25	3.175	16.5
	40	18.606	6.850	36.81
	70	15.740	2.965	18.84
Biotin-deficient	120	9.775	6.300	64.45
	168	6.870	6.600	96.05
Biotin-deficient + Oleic acid	96	9.770	3.540	36.23
	216	9.200	7.400	80.42
Biotin-deficient + Aspartic acid	69	16.150	1.900	11.77
	115	11.220	2.425	21.63
Biotin-deficient + Aspartic acid + Oleic acid	72	17.800	3.750	21.07
	96	13.830	3.050	22.05

Yeast was grown in media containing an optimal concentration of biotin, a suboptimal concentration of biotin or a suboptimal concentration of biotin and supplemented with oleic acid (100 µg./ml.), or aspartic acid (2.0×10^{-3} M) or a mixture of aspartic

TABLE XVIII (contd.)

acid + oleic acid. Yeast was harvested by centrifugation, washed and 100 mg. dry weight of yeast suspended in 5.0 ml. of ice-cold water and disrupted in a Mickle disintegrator with 3.0 g. of glass beads for ½ hr. The protein content of the whole cells was determined by the Kjeldahl technique while the protein content in cell-free extracts was estimated by the method of Lowry *et al.* (1951).

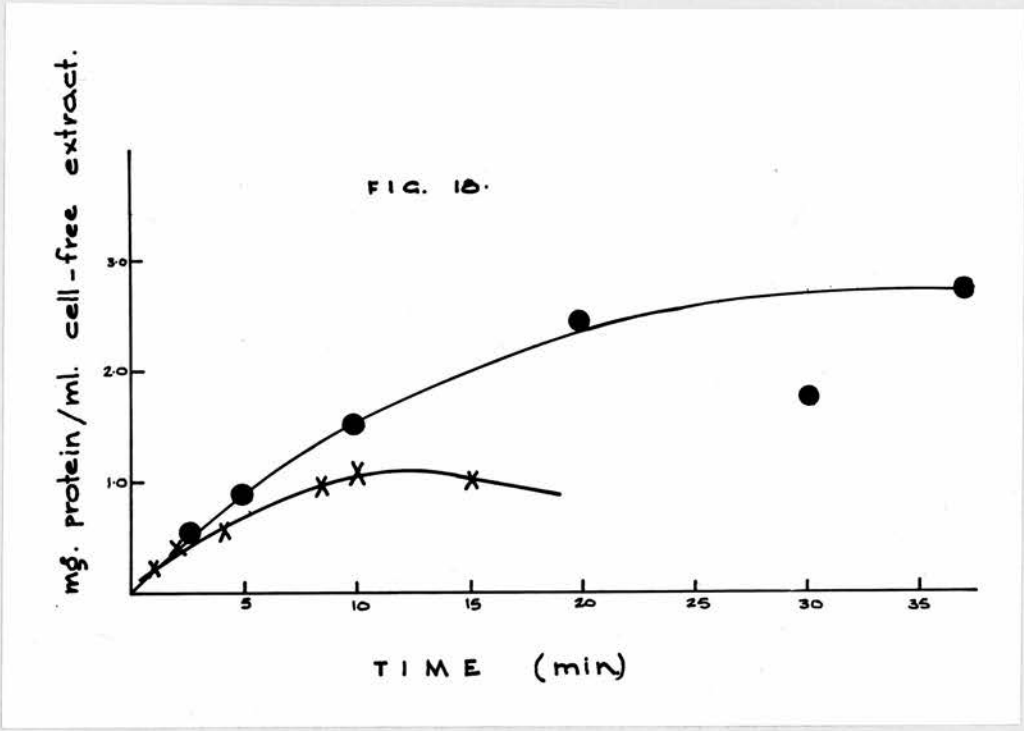


Figure 18

Disruption in the Mickle disintegrator of yeast grown in media containing an optimal (8.0×10^{-10} M) or a suboptimal (0.4×10^{-10} M) concentration of biotin. The biotin-optimal yeast (●——●) was 40 hr. old and the biotin-deficient yeast (X——X) was 120 hr. old. The yeast cells obtained by centrifuging the cultures, were washed thrice with ice-cold water and then disrupted by suspending 100 mg. dry wt. of the yeast in 5.0 ml. of ice-cold water containing 3.0 g. of glass beads. The extracts were centrifuged and the protein content determined by the method of Lowry et al. (1951). Duration of treatment of the yeast in the Mickle disintegrator is plotted against mg. protein/ml. of cell-free extract.

b) Variations in enzyme activity during growth of the yeast in biotin-optimal medium: The data in Fig. 19 show the variations of each of the four enzymes during growth of the yeast in unsupplemented biotin-optimal medium. These data show that the invertase activity of whole cells and cell-free extracts increased rapidly during the exponential phase of growth, and continued to increase when the yeast entered the stationary phase of growth. The activity in whole cells increased at a very much greater rate than that in cell-free extracts, which suggested that, as the yeast aged, an increasing proportion of the invertase activity was concentrated in the cell wall. The malic dehydrogenase activity of the cell-free extracts, like the invertase activity, increased rapidly from a very low value in early exponential-phase yeast to higher values in late exponential-phase yeast. However, the activity of malic dehydrogenase, unlike that of invertase, declined slightly during the stationary phase of growth. The exponential phase of growth also saw an increase in the CPOC transferase activity, the rate of increase being most rapid during the early exponential phase. On the other hand, the acid pyrophosphatase activity of whole cells declined as the culture aged, the activity being greatest during the early exponential phase of growth and declining to a low value in stationary phase yeast.

Figure 19

Variations in the activities of acid pyrophosphatase (⊕------⊕) and invertase (○———○) in whole cells, and of CPOC transferase (△———△), invertase (◕———◕) and malic dehydrogenase (×———×) in cell-free extracts of the yeast during growth (●———● , mg. dry wt./ml.) in biotin-optimal medium. Acid pyrophosphatase activity is expressed as μM ATP dephosphorylated/mg. cell protein/hr., CPOC transferase as mM ornithine converted/μg. extract protein/hr./100, invertase as μM sucrose hydrolysed/mg. extract or cell protein/hr./1000, and malic dehydrogenase as μM malate dehydrogenated/μg. extract protein/min.

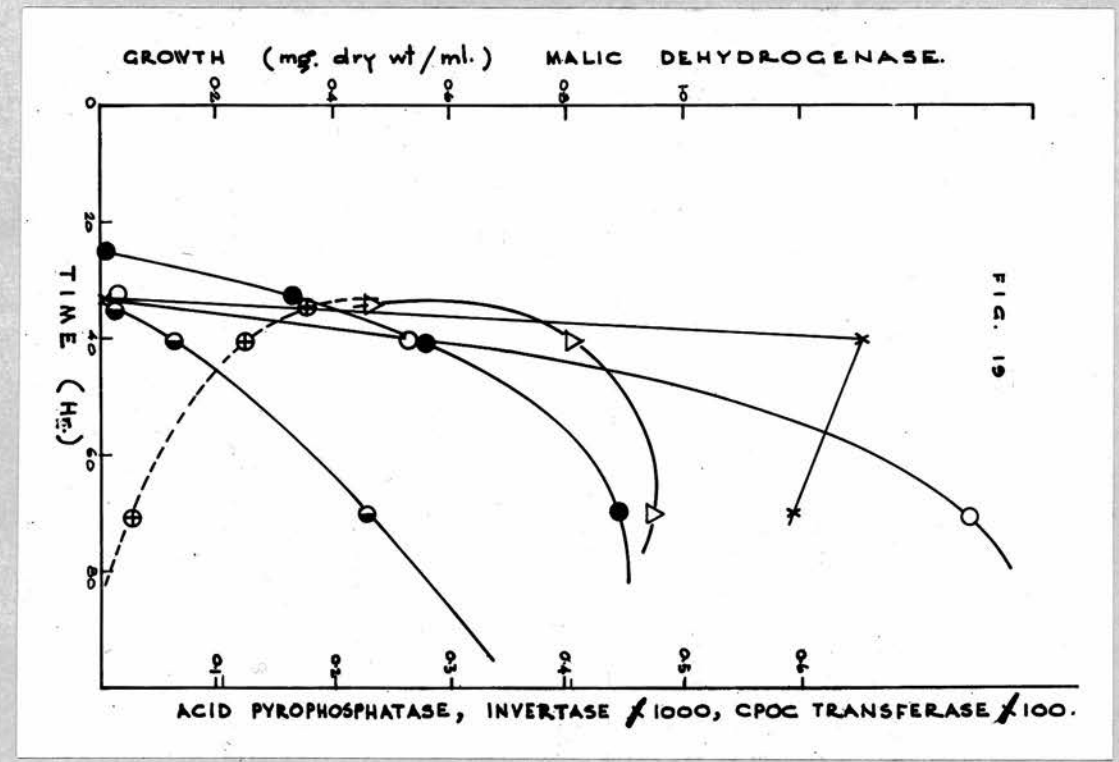

GROWTH (mgs. dry wt/ml.)
MALIC DEHYDROGENASE.
TIME (Hrs.)
ACID PYROPHOSPHATASE, INVERTASE /1000, CPOC TRANSFERASE /100.

FIG. 19

c) Enzyme activities of yeast grown in unsupplemented biotin-deficient medium and in biotin-deficient media supplemented with various biotin-sparing compounds: Cultures of the yeast grown in biotin-deficient medium do not show sharply defined exponential and stationary phases of growth that are seen in biotin-optimal cultures (Fig. 20). Nevertheless, the yeast growth in biotin-deficient medium supplemented with aspartic acid is stimulated to some extent and is accompanied by a marked restoration of nucleic acids and protein synthesis. Stimulation of growth in biotin-deficient media supplemented with oleic acid is much slower, and is not accompanied by increased nucleic acids and protein synthesis. When, however, biotin-deficient medium is supplemented with aspartic acid and oleic acid, the sequence of changes in the nucleic acid and protein content of the yeast is qualitatively similar to that which occurs in biotin-optimal yeast; also, under these conditions, the yeast grows rapidly with cultures showing well defined exponential and stationary phases of growth (Fig. 20).

The data in Table XIX show the enzyme activities of yeast grown, for varying periods of time, in unsupplemented biotin-deficient medium and in biotin-deficient medium supplemented with L-aspartic acid or oleic acid or both of these compounds.

It can be seen that, during growth of the yeast in

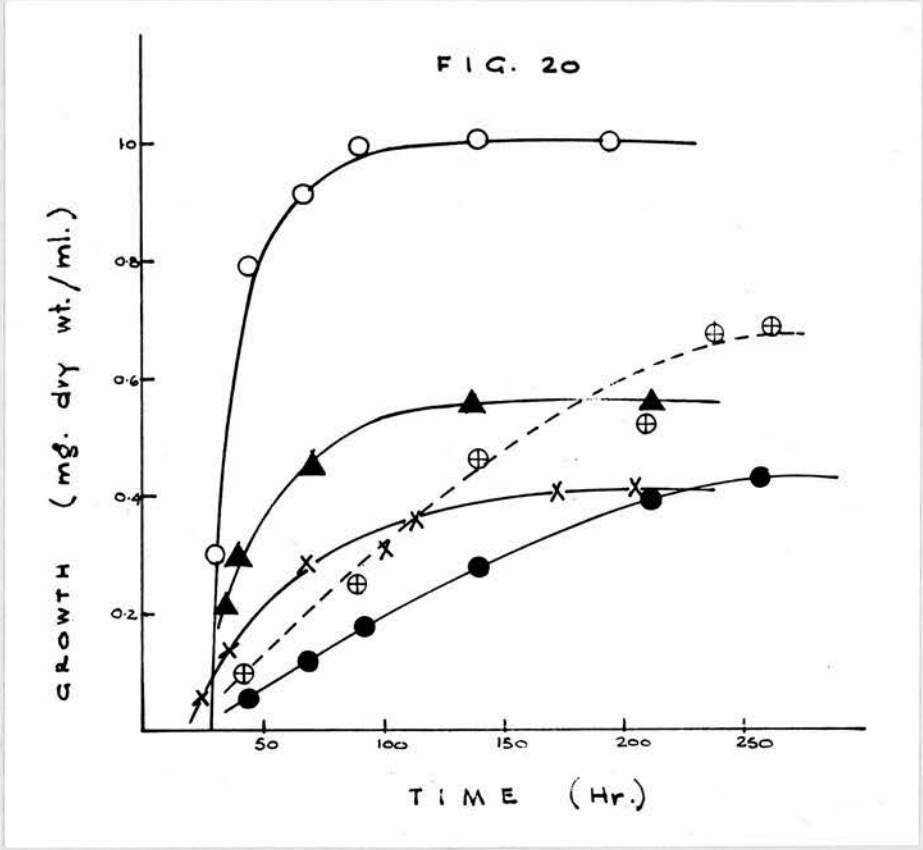


Figure 20

Effect of incubation time on growth of the yeast in media containing an optimal (8.0×10^{-10} M) concentration of biotin (○——○), a suboptimal (0.4×10^{-10} M) concentration of biotin (●——●), and a suboptimal concentration of biotin but supplemented with L-aspartic acid (2.0×10^{-10} M) (×——×), oleic acid (100 µg./ml.) (⊕-----⊕) or a mixture of aspartic acid + oleic acid (▲——▲).

TABLE XIX

Activities of invertase (whole cells and cell-free extracts), acid pyrophosphatase, carbamyl phosphate ornithine carbamyl transferase (CPOC transferase) and malic dehydrogenase in, yeast grown in media containing an optimal and a suboptimal concentration of biotin and in, yeast grown in media containing a suboptimal concentration of biotin but supplemented with aspartic acid (2.0×10^{-3} M), oleic acid (100 µg./ml.) or aspartic acid + oleic acid

Medium in which yeast was grown	Age (hr.)	Growth (mg. dry wt./ml.)	Invertase (µmoles sucrose hydrolysed/mg. protein/hr.)		Acid pyrophosphatase (µmoles ATP hydrolysed/mg. protein/hr.)	Malic dehydrogenase (µmoles malate dehydrog./µg. protein per min.)	CPOC transferase (mmoles ornithine reacted/mg. protein/hr.
			W-cells	C-free ext.			
Biotin-optimal	33	0.34	5.37	4.25	0.181	trace	23.4
	40	0.73	270.00	61.77	0.123	1.31	40.6
	70	0.89	744.60	230.10	0.051	1.19	47.3
Biotin-deficient	120	0.200	723.70	241.00	nil	0.76	29.4
	168	0.350	1583.20	528.00	0.277	3.04	28.5
Biotin-deficient + oleic acid	96	0.290	500.00	197.50	0.210	1.23	21.7
	216	0.580	1391.70	1056.00	0.081	4.76	24.5
Biotin-deficient + L-aspartic acid	69	0.290	22.90	15.80	1.880	1.43	12.7
	115	0.360	72.90	30.60	2.850	3.23	15.1
Biotin-deficient + L-aspartic acid + oleic acid	72	0.550	112.70	62.80	2.670	2.17	12.5
	96	0.705	353.10	247.40	3.120	3.45	14.1

Enzyme assays were carried out as detailed under METHODS.

unsupplemented biotin-deficient medium, the activities of acid pyrophosphatase, invertase and malic dehydrogenase increased to values higher than those recorded in biotin-optimal yeast. The CPOC transferase activity, however, remained lower than that recorded in late exponential phase and stationary phase biotin-optimal yeast. The pattern of enzyme activities in yeast grown in biotin-deficient medium supplemented with oleic acid was similar to that observed in yeast grown in unsupplemented biotin-deficient medium, although the acid pyrophosphatase activity declined rapidly as the culture aged. The CPOC transferase activity of oleic acid supplemented yeast was also low. Yeast grown in biotin-deficient media supplemented with aspartic acid, with or without oleic acid, showed a pattern of enzyme activities quite different from that in yeast grown in aspartate-free biotin-deficient medium. The acid pyrophosphatase activity of aspartate-supplemented yeast increased to values much higher than those recorded even in biotin-optimal yeast, while the invertase activities correspondingly diminished. This decline in invertase activity was most marked in biotin-deficient medium supplemented with only aspartic acid. The malic dehydrogenase activity of aspartate supplemented yeast was also higher than that recorded in biotin-optimal yeast. Also, the CPOC transferase activity in yeast grown in aspartate-containing biotin-deficient medium was

below that of early exponential phase biotin-optimal yeast.

There was no detectable change in activity of any of the enzymes in whole cells or in cell-free extracts when the reaction mixtures were supplemented with biotin (16.0×10^{-10} M). This indicated that the changes observed in the activities of various enzymes were not the direct result of a deficiency of biotin.

CONCLUSIONS

1. Studies on the activities of the enzymes acid pyrophosphatase, invertase, malic dehydrogenase and carbamyl phosphate ornithine carbamyl transferase (CPOC transferase) showed that the syntheses of these enzymes were probably biotin-independent except in the case of CPOC transferase, in which case a direct involvement of biotin would seem possible.
2. Yeast grown under conditions of biotin deficiency was shown to possess comparatively fragile cell walls, probably a reflection of an alteration in the chemical composition of the walls. This fragility might be explained on the basis of a lower protein content in the cell walls of yeast grown under conditions of biotin deficiency.

DISCUSSION

DISCUSSION

Three main lines of attack have been used in the elucidation of the metabolic function of B group vitamins. The first of these involves studying the metabolic behaviour of vitamin-dependent organisms under conditions of relative vitamin deficiency. In single-celled organisms, the lack of an essential factor (e.g. a B group vitamin) results in retardation of growth. It is possible, therefore, to study the metabolic derangements caused by the vitamin deficiency in micro-organisms and thus deduce possible biochemical functions of the vitamin. This line of approach has been used by several workers and is said to yield better results in higher plants and animals than in unicellular micro-organisms.

A second method entails the use of the competitive analogue-metabolite inhibition technique. This technique has been applied to growth studies with micro-organisms and has also been used to advantage in work on mammalian metabolism. The competitive analogue-metabolite inhibition technique is a development of the observation (Woods, 1940), that the bacteriostatic effect of sulphanilamide is competitively reversed by the structurally related compound p-aminobenzoic acid, a compound not previously known to

possess any biochemical function. It was suggested that sulphanilamide, a structural analogue of p-aminobenzoic acid, served as a competitive inhibitor of an enzyme system for which p-aminobenzoic acid was an essential co-factor. Later, this discovery prompted a wide search for chemotherapeutic agents among structural analogues of compounds known to have metabolic importance. The application of competitive analogue-metabolite inhibition technique to the study of microbial metabolism was pioneered largely by Shive and his collaborators (Williams, Eakin, Beerstrecher & Shive, 1950).

A third method involves the isolation of an enzyme and determination of the vitamin content of the enzyme during various stages of purification procedure. The discovery of a correlation between enzymic activity and vitamin content is taken to suggest that the vitamin is essential for enzyme activity. Many examples can be given where vitamins belonging to B group have been found to occur as co-enzymes. These include thiamin, which occurs bound to an enzyme (carboxylase), and riboflavin which in the form of flavin mononucleotide and flavine adenine dinucleotide is involved in electron transport.

Each of the above methods for studying the metabolic functions of vitamins has its merits and demerits. Only the first of these methods has been used in the studies on biotin function in Saccharomyces cerevisiae reported in

this thesis. Conflicting reports are found in the literature following the use of the deficiency method, and this is especially true of studies on biotin function. This may be due to the use of different suboptimal concentrations of the vitamin by various workers, and this is why the conditions used in the present study were carefully controlled, especially with regard to the concentration of biotin in suboptimal media. At concentrations below the suboptimal level (0.4×10^{-10} M), growth of the yeast was very small; this minimum concentration was necessary in order to obtain sufficient material for analyses.

The work reported in this thesis has shown that biotin deficiency has several profound effects on the chemical composition of Saccharomyces cerevisiae. The principal disturbances observed under conditions of biotin deficiency in the yeast were an impairment in the synthesis of nucleic acids and total protein and variations in the activities of certain enzymes. In this discussion an attempt will be made to relate these findings to the hypotheses put forward concerning the metabolic functions of biotin. An account of these hypotheses has been given in the INTRODUCTION.

Since purine- and pyrimidine-containing nucleotides (e.g. ATP, DPN and co-enzyme A) and polynucleotides

(nucleic acids) are essential components of all living cells, it is to be expected that any metabolic stress which causes a derangement in the biosynthetic processes leading to the formation of purines or pyrimidines will result in the decreased synthesis of nucleotides and nucleic acids. Although the presence of diminished amounts of total purines in biotin-deficient micro-organisms has not been demonstrated directly, several workers have obtained evidence that biotin is concerned in the synthesis of these nitrogenous bases. The effect of this biotin-conditioned purine deficiency on the synthesis of certain nucleotides in biotin-requiring micro-organisms has already been reported. Thus Katsuki (1959, a & b), has shown that biotin-deficient Piricularia oryzae and Bacillus macerans contained diminished amounts of ATP, DPN and Co A, and Rose (1960, a & b) reported that the excretion of nicotinic acid and nicotinic acid adenine dinucleotide, two biosynthetic precursors of pyridine nucleotides which appear in the culture medium during growth of Saccharomyces cerevisiae under conditions of biotin deficiency, is suppressed on adding adenine to the medium. During the course of the present study, a decrease in the ATP content of the yeast under conditions of biotin deficiency was observed, thus confirming the findings of Katsuki (1959, a & b) and Briggs (personal communication); addition of adenine restored synthesis of ATP (Table 10). This restoration of ATP

synthesis on adding adenine to the biotin-deficient medium probably explains the results reported by Rose (1960, b), who suggested that excretion of these intermediates in DPN synthesis occurred as a result of a deficiency of ATP.

Data given in Section 1(a) of RESULTS clearly demonstrate that, under conditions of biotin deficiency, growth of the yeast is adversely affected and the contents of nucleic acid, and protein are diminished. Also, the yeast produces a pink pigment when grown under conditions of biotin deficiency.

It was rather surprising to discover that, during the early stages of growth, the RNA in the biotin-deficient yeast contained an abnormally high content of purine, in view of the adverse effect of biotin deficiency on purine biosynthesis (Tables VIII, IX). There was further evidence of a difference between the RNA from 5-day biotin-deficient culture and from 7-day biotin-deficient and from biotin-optimal cultures, in that the first of these was readily hydrolysed by N-perchloric acid (Fig. 4). This observation is similar to that reported by Horowitz, Lombard and Chargaff (1958), who found an instability in the RNA produced in the presence of chloramphenicol in Escherichia coli; however, these workers could not find any difference in the base ratio between the unstable RNA and the normal RNA in this bacterium. The suggestion that the RNA produced in the presence of chloramphenicol (which

is known to arrest protein synthesis) probably represents an intermediary stage in an obligatory pathway to a more complex subatance (e.g. a nucleoprotein that is less susceptible to degradation) is of interest, since the instability of RNA observed in the present investigation was accompanied by a cessation in protein synthesis. The suggestion that the RNA produced under conditions of biotin deficiency is probably non-functional cannot be ruled out, for this could explain an impairment in protein synthesis. It is, however, important to note that the RNA extracted by the method used in the present study was heterogeneous and consisted of a mixture of all the types of RNA present in the yeast. The slight difference in the overall base ratio might then be caused by a more significant variation in the base ratio of these RNA fractions, since a diversity in base ratios has been reported among different types of RNA in yeast (Monier, Stephenson & Zamecnik, 1960).

This decrease in the amounts of nucleic acids synthesized under conditions of biotin deficiency was accompanied, during the early stages of growth, by a significant increase in the concentration of intracellular ultraviolet-absorbing substances which, since they absorbed maximally at or very close to 260 mu (Figs. 7 & 8), were taken to be purine- or pyrimidine-containing compounds. These compounds were detected initially in extracts made with strong acids (perchloric and trichloroacetic acid) and

it was thought probable that they might represent products from the acid degradation of RNA and DNA. When it was found, however, that these ultraviolet-absorbing substances were also extracted, though more slowly, with aqueous n-butanol at pH 4.5, a much less drastic reagent, then it was assumed that they did not represent artefacts of extraction. Some supporting evidence for this contention came from the finding that the amounts of ultraviolet-absorbing substances did not increase significantly when the temperature of extraction was raised from 3° to 21° or 30°, as might have been expected had they arisen as a result of hydrolysis of nucleic acids. No analyses of the composition of this acid-soluble fraction were made in the course of the present investigation, so it is not known what type of purine- or pyrimidine-containing compounds were present. However, at least two ultraviolet-absorbing purine precursors, 5-amino imidazole riboside and hypoxanthine or inosine (Lones, Rainbow & Woodward, 1958; Moat et al., 1956), are known to be excreted by Saccharomyces cerevisiae growing under conditions of biotin deficiency, so that it is likely that these also accumulate intracellularly under conditions of decreased RNA synthesis. This has been demonstrated, for example, for a strain of Escherichia coli following addition to the culture of the purine analogue 6-azauracil (Skoda & Sorm, 1958). As pointed out in the INTRODUCTION, there exists a close relationship between RNA

synthesis and protein synthesis and, on this basis, the restriction in protein synthesis under conditions of biotin deficiency might be attributed to: (a) the presence of diminished amounts of ATP in the yeast under conditions of biotin deficiency, for one of the functions assigned to ATP is its capacity to activate amino acids in protein synthesis; (b) the presence of decreased amounts of amino acids, the building blocks of proteins; and (c) the lack of the required amounts of RNA, which has been suggested to act as a template or mould for protein synthesis.

A deeper probe into this restriction of growth, RNA and protein synthesis under conditions of biotin deficiency was undertaken by studying the effects of certain biotin-sparing substances on the synthesis of nucleic acids, protein and related substances by the yeast. It was hoped that this line of attack might yield further information as to the possible involvement of biotin in specific areas of yeast metabolism. It can be seen from the results obtained that the biotin-sparing action of oleic acid, together with that of other fatty acids present in the sample of oleic acid used, was probably fundamentally different from the effect brought about by

aspartate, casamino acids, adenine or adenosine. The growth-promoting effect of this fatty acid was exhibited much more slowly than that caused by the other biotin-sparing substances tested, and it was not accompanied by an increased synthesis of nucleic acids and protein. Longer periods of incubation did result in increased growth but did not bring about any increase in the contents of nucleic acids, protein and related substances in the yeast. A further difference between the biotin-sparing action of oleic acid and that of the other substances tested (with the exception of adenosine) was that yeast grown in media supplemented with oleic acid remained pink in colour, whereas the addition of adenine, aspartate or casamino acids in biotin-deficient media suppressed the formation of pink pigment. The chemical nature of this pink pigment is not yet known, but its production has been reported to be associated with an impairment in the metabolic processes leading to purine biosynthesis (Chamberlain, Cutts & Rainbow, 1952). This supported the finding that, in yeast grown in biotin-deficient medium containing oleic acid, there was no appreciable restoration of purine synthesis. Still further evidence to support the suggestion that the biotin-sparing action of oleic acid differed from that of the other substances tested came from the observation of a synergistic action in the ability of a mixture of aspartic and oleic acids to spare the

growth-promoting action of biotin. The marked action of a mixture of aspartic acid and oleic acid has been noted by Potter and Elvehjem (1948), who reported that this mixture of compounds could almost completely replace biotin in the nutrition of Lactobacillus arabinosus. These workers also found that the biotin requirement of L. arabinosus in the absence of aspartic acid was ten times as high as in its presence. They put forward the suggestion that the transamination of oxaloacetic acid was not a limiting factor in the slow growth caused by biotin deficiency, and this is interesting in view of the report by Lichstein and Christman (1948) that, in Bacterium cadaveris, biotin functions in the reversible deamination of aspartic acid.

Previous workers (Chamberlain, Cutts & Rainbow, 1952; Moat, Wilkins & Friedman, 1956) have suggested that the biotin-sparing action of aspartic acid is explained largely by its ability to restore purine synthesis, the amino acid functioning both in the synthesis of inosinic acid (Wahba & Shive, 1954) and in amino group transfer within the purine skeleton (Abrams & Bentley, 1955). The data obtained during this investigation substantially support this view for, in yeast grown in media containing either aspartic acid alone or casamino acids, there was a marked increase in the RNA content as compared with yeast grown in amino acid-free media. The finding that casamino

acids had a higher stimulatory effect on protein synthesis and growth of the yeast, as compared with aspartic acid alone, suggests that, although aspartic acid can bring about a substantial restoration of RNA synthesis, it is necessary to provide the organisms with additional exogenous amino acids for this increased RNA synthesis to lead to synthesis of additional protein. Thus, the pools of intracellular amino acids and acid-labile phosphate in yeast grown in biotin-deficient medium supplemented with aspartic acid alone fell to a very low value. This could be explained by an inability on the part of biotin-deficient yeast to synthesize other amino acids from aspartic acid, for biotin has been reported to be essential in certain transamination reactions (Lichstein & Umbreit, 1947; Lichstein & Christman, 1948; Nadkarni & Sreenivasan, 1957). The decrease in acid-labile phosphate caused by the addition of aspartic acid, casamino acids or a mixture of oleic acid + aspartic acid or casamino acids, could possibly be due to the higher rate of synthesis of phosphorylated compounds (RNA and DNA), and also the inability of the yeast grown under these conditions to replenish the phosphate pool.

The biotin-sparing action of adenine and adenosine would also seem to depend upon their inability to circumvent the metabolic lesion in purine synthesis induced by biotin deficiency, although there is apparently a bio-

chemical difference between the actions of these two compounds since adenine, but not adenosine, suppressed the formation of pink pigment by the yeast. This effect could presumably be due to the fact that, when adenine is added exogenously to the biotin-deficient medium, the sequence of enzymic reactions leading to the synthesis of this purine (at the nucleotide level) is repressed, the result being that the precursors of adenine which accumulate under conditions of biotin deficiency and give rise to the pink pigment are no longer accumulated and therefore, the yeast appears creamy white. When adenosine is included in the biotin-deficient medium the yeast still develops a pink colour, possibly because adenosine is incapable of repressing the reactions leading to purine synthesis, as it cannot be converted to inosinic acid or adenylic acid.

Biotin is known to be essential for the synthesis of fatty acids, and a co-enzymic role for this vitamin in the carboxylation of acetyl-Co A during fatty acid synthesis has recently been demonstrated (Wakil, 1961). Cells of Saccharomyces cerevisiae contain about 4% by weight of lipid (Newman & Anderson, 1933) and, since it is likely that most of this lipid exists in the form of membranes, biotin-deficient yeast, which is probably unable to synthesize certain fatty acids, must contain less membranous material as compared with biotin-optimal yeast. The effects of this shortage of membranous material on the

biochemical organisation of the yeast cell must be profound. It could mean that additional protein synthesized by organisms in biotin-deficient media containing adenine, adenosine or amino acids cannot become functional because of a lack of suitable membranes on which certain of these enzymes become orientated. The importance of these membranes in relation to protein synthesis has been discussed by Gros (1960). It has been suggested, that perhaps only those ribonucleoprotein particles physiologically associated with the cell membranes are capable of completing the protein synthetic sequence (Hoagland, 1960). In mammalian cells too, the most active incorporation of labelled amino acids occurs in microsomes which are, in essence, particles with attached membranes. In disrupted bacterial cells, incorporation of amino acids takes place without a net increase in protein content, which it has been suggested is owing to an irreversible separation of cell membranes without which the synthesized protein cannot be removed from the site of synthesis. In addition, lipo-amino acid complexes have been reported to act as intermediates in the synthesis of proteins by micro-organisms (Hunter & Goodsall, 1961). The evidence provided above supports the suggestion that any impairment in the lipid-synthesizing capacity of the yeast under conditions of biotin deficiency might impose a further restriction on protein synthesis. The discovery of a synergistic effect

of a mixture of oleic acid and aspartic acid under conditions of biotin deficiency lends support to the importance of membranes in protein synthesis by the yeast, because RNA synthesis was restored to an appreciable extent when aspartic acid was included in the biotin-deficient medium but was without any appreciable effect on the growth. On the other hand, oleic acid was ineffective in restoring RNA synthesis, but it stimulated growth of the yeast under conditions of biotin deficiency. However, when a mixture of aspartic acid and oleic acid was included in the biotin-deficient medium, not only was RNA synthesis restored to an appreciable extent but growth was stimulated as well. This evidence, which is indirect, lends support to the idea of the importance of lipids in protein synthesis. In all probability, these lipid membranes are essential in mobilising the proteins formed on the template and orientating them on sites where they can discharge their enzymic functions.

However, even in biotin-deficient medium supplemented with comparatively high concentrations of aspartic acid or casamino acids and oleic acid, growth of the yeast was still appreciably restricted as compared with that in biotin-optimal medium; growth in biotin-free medium supplemented with either of these pairs of biotin-sparing substances was even more severely restricted. Since aspartic acid and casamino acids were each shown to be

capable of restoring synthesis of RNA and protein to an appreciable extent, it must be concluded that utilisation of this protein in metabolic reactions leading to growth of the yeast is dependent upon a supply of biotin which cannot be replaced by the biotin-sparing compounds examined in this study. The nature of these particular biotin-requiring reactions is not known, but there is evidence to suggest that they may be concerned with the synthesis of the yeast cell wall constituents. Cells of Saccharomyces cerevisiae grown in biotin-deficient medium contain increased amounts of glucan and diminished amounts of mannan as compared with biotin-optimal yeast; both of these polysaccharides are known to occur in the cell walls of Saccharomyces cerevisiae (Northcote & Horne, 1952). The polysaccharide composition of yeast grown in biotin-deficient media supplemented with aspartic acid, or with aspartic acid + oleic acid, showed even greater differences in the contents of glucan and mannan and, under these conditions, the organisms grew in large aggregates. Aggregation of yeast cells under these conditions is probably responsible for restricting cell division and so may be partly responsible for the decreased growth. Further evidence that biotin functions in the metabolism of yeast cell wall constituents comes from the report by Nickerson (1961) that, in Candida albicans, biotin is concentrated in the cell wall glucomannan-protein complex.

It is perhaps significant that biotin should be associated with a cell wall component that has been shown to be synthesized in restricted amounts under conditions of biotin deficiency.

It would seem therefore that, in yeast, biotin is concerned in three major areas of metabolism, namely in the synthesis of nucleic acids and protein mainly through its role in aspartic acid synthesis, in the formation of lipid membranes through its involvement in unsaturated fatty acid synthesis, and in the metabolism of certain cell wall constituents through an as yet unknown mechanism.

During the last part of the present study an attempt was made to discover the effects of biotin deficiency on the activities of certain enzymes namely, acid pyrophosphatase, invertase, malic dehydrogenase and carbamyl phosphate ornithine carbamyl transferase (CPOC transferase). It was possible that the diminution in the amount of protein synthesized in biotin-deficient yeast could affect only certain enzymes, which would explain why only a limited number of enzymic activities have been reported to be impaired in micro-organisms grown under conditions of biotin deficiency. On the other hand, the decrease in protein synthesis might have a non-specific

effect on enzyme synthesis, with the result that production of all of the enzymes in the cells was decreased to about the same extent; then the deficiency might only be observed in those metabolic reactions for which the enzymes were present in rate-limiting concentrations. The activities of these four enzymes were taken as parameters for the synthesis of various types of protein. Thus these four enzymes are known to be located on different organelles within the cell. Malic dehydrogenase is a particulate enzyme, while CPOC transferase occurs in soluble fraction. Both invertase and the acid pyrophosphatase studied are cell wall enzymes.

From the results reported in Section III of RESULTS, it would seem that biotin deficiency does not have any specific effect, direct or indirect, on the synthesis of acid-pyrophosphatase, invertase or malic dehydrogenase by the yeast, for the activities of these enzymes were higher in biotin-deficient yeast than in yeast at any stage of growth in biotin-optimal medium; in yeast grown in the presence of the biotin-sparing substances aspartic acid and oleic acid, the activities of these enzymes were often greater than in yeast grown in unsupplemented biotin-deficient medium. The activity of CPOC transferase however, never reached the level in biotin-deficient yeast that was observed in late exponential phase and stationary phase biotin-optimal yeast. Moreover,

in yeast grown in media supplemented with aspartic acid, with or without oleic acid, CPOC transferase activity was, in general, lower than in early exponential phase biotin-optimal yeast. It would appear, therefore, that biotin deficiency may have a direct or indirect effect on the synthesis of this particular enzyme.

MacLeod, Grisolia, Cohen and Lardy (1949) first reported that biotin deficiency in rat liver homogenates resulted in a loss in the ability to convert ornithine to citrulline, and that this deficiency could not be restored with supplements of biotin. Subsequently, Ravel, Grona, Humphreys and Shive (1959) concluded that biotin is concerned indirectly in the synthesis of CPOC transferase in Streptococcus lactis. The observations made during the present study are in agreement with this conclusion and, since the activity of the enzyme was followed throughout growth of the yeast in biotin-optimal medium, they provide more vigorous evidence.

Synthesis of enzymes during growth of micro-organisms is thought to be controlled by an interplay of induction and repression mechanisms (Pardee, 1959). One possibility therefore is, that under conditions of biotin deficiency synthesis of CPOC transferase is permanently repressed. Although direct evidence to refute this hypothesis is not available, it is, on the basis of the evidence at hand, an unlikely explanation. Thus, repression was observed

in yeast in many different physiological states. Moreover, certain of the factors controlling synthesis of CPOC transferase have been reported (Gorini & Maas, 1957). It is known, for instance, that arginine in certain bacteria can suppress synthesis of this enzyme, while in other bacteria this amino acid is capable of inducing synthesis of CPOC transferase (Gorini & Gundersen, 1961). But, CPOC transferase synthesis was suppressed in yeast containing amino acid pools of various sizes. It seems likely therefore, that biotin may have a specific role in the synthesis of CPOC transferase although in the absence of more complete data concerning the mechanism controlling induction and repression of this enzyme, the possibility of a non-specific effect cannot be precluded.

The effect of biotin deficiency on the activities of acid pyrophosphatase, invertase and malic dehydrogenase might well be explained on the basis of an alteration in the controlling induction and repression mechanisms in yeast grown under the stress of biotin deficiency. McCarthy and Hinshlewood (1959) studied the variations in phosphatase activity during the growth cycle of Bacterium lactis aerogenes and found a rapid increase in the amount of this enzyme in each cell during the early stages of growth and a decline during the later stages of growth. The changes in the acid pyrophosphatase activity of the yeast grown in biotin-optimal medium followed a similar

pattern to that reported by McCarthy and Hinshlewood for Bacterium lactis aerogenes. It has been reported that synthesis of alkaline phosphatase in Escherichia coli is suppressed by inorganic phosphate. A similar type of suppression mechanism might be operating in yeast, for in yeast grown in aspartate supplemented biotin-deficient medium, in the presence or absence of oleic acid, the content of acid-labile phosphate was low while the acid pyrophosphatase activity was extremely high as compared with biotin-optimal yeast.

A sequence of changes in malic dehydrogenase activity similar to that observed in biotin-optimal yeast has been reported by Wooldridge, Knox and Glass (1936) for dehydrogenases in other micro-organisms. Yeast grown in supplemented or unsupplemented biotin-deficient media had comparatively high malic dehydrogenase activities as compared with biotin-optimal yeast. Although repression studies on this enzyme have not yet been reported, it is possible that synthesis of this enzyme is repressed by the end product of the reaction, oxalo-acetate. This could explain the high activities in biotin-deficient yeast, for synthesis of oxaloacetate is known to be biotin-dependent with biotin possibly functioning co-enzymically.

The invertase activity of yeast has been shown by several workers to increase with increasing age of culture

(DeLey & Vandamme, 1950; Davies, 1956; Suomalainen & Oura, 1957), and the results obtained fully substantiate these findings. DeLey and Vandamme suggested that the invertase activity of the yeast cell was greatest when the cells had their lowest biochemical activity, e.g. during stationary phase of growth. It might appear then that active protein synthesis represses invertase synthesis, and this could help to explain the low invertase activities in yeast grown in aspartate containing biotin-deficient medium, for under these conditions protein synthesis in biotin-deficient yeast has been shown to be stimulated. It has also been reported that glucose represses invertase production (Davies, 1953). Since invertase activity is high in biotin-deficient yeast, it would appear that any repression attributed to glucose is caused by the products of glucose metabolism rather than by the sugar itself for, in biotin-deficient cultures there are considerable amounts of residual glucose while glucose catabolism is diminished.

Previous workers have shown that biotin deficiency causes changes in the synthesis of cell wall polysaccharides in Saccharomyces cerevisiae (Dunwell, Ahmad & Rose, 1961). Further evidence of a derangement in cell wall metabolism in biotin-deficient yeast was seen in the extreme fragility of yeast grown in unsupplemented biotin-deficient medium. Addition of aspartic acid and oleic

acid to the biotin-deficient medium enabled the yeast to grow for a time as rapidly as in biotin-optimal medium. But, addition of these compounds to biotin-deficient medium is known to cause still further differences in the glucan and mannan contents of the yeast (Dunwell *et al.*, 1961) although, as shown in this study, it renders the cells more resistant to mechanical breakage probably because the cell wall contains more protein. It is possible, therefore, that biotin is involved directly in the synthesis of certain enzymes concerned with yeast cell wall metabolism. But, from the results reported herein, neither acid pyrophosphatase nor invertase would appear to be a cell wall enzyme, synthesis of which is biotin-dependent.

It can be seen that the results reported in this thesis have provided evidence which supports roles for biotin in three quite different areas of yeast metabolism, namely in synthesis of nucleic acids and protein, production of lipids, and some as yet unknown function in cell wall metabolism. It would appear that the biotin-conditioned derangements which affect the synthesis of these cell constituents are probably secondary effects, a reflection of the inability of the yeast to carry out

certain carboxylation and decarboxylation reactions in which biotin functions co-enzymically. Of the functions assigned to biotin, that concerned with cell wall metabolism is least understood in yeast. Further progress in elucidating the role of biotin in yeast metabolism will require extensive studies on the role of this vitamin in the synthesis of cell wall constituents. It would also be interesting to discover other enzymes (like carbamyl phosphate ornithine carbamyl transferase) synthesis of which is specifically biotin-dependent.

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APPENDIX 1

A study of the effect of biotin on the aggregation of yeast cells grown in a biotin-deficient medium containing L-aspartic acid, casamino acids or a mixture of L-aspartic acid or casamino acids + oleic acid was undertaken in this laboratory by Mr. J.L. Dunwell. During this investigation, the author assisted Mr. Dunwell in the experimental work. The results obtained form the subject of a communication published in Biochim. Biophys. Acta, 51, 604 (Dunwell, Ahmad & Rose). A copy of this paper is appended as well.

ACKNOWLEDGEMENTS

The author takes this opportunity to express his gratitude to Dr. A.H. Rose, Ph.D., and Prof. I.A. Preece, D.Sc., F.R.S.E., for their advice, help, supervision and encouragement throughout the period of the present study. The author also owes a deep debt of gratitude to Dr. A.H. Rose for his technical assistance. Supervision and helpful suggestions of Prof. E.L. Hirst, D.Sc., F.R.S., are also gratefully acknowledged.

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Effect of Biotin Deficiency on the Synthesis of Nucleic Acids and Protein by *Saccharomyces cerevisiae*

BY FAZAL AHMAD, A. H. ROSE AND N. K. GARG*

Microbiology Laboratory, Department of Applied Biochemistry, Heriot-Watt College, Edinburgh, Scotland

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SUMMARY

A strain of *Saccharomyces cerevisiae*, grown in a medium containing a suboptimal concentration ($0{\cdot}4 \times 10^{-10}$ M) of biotin, was shown to contain less deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein but, during the early stages of growth, increased concentrations of acid-soluble ultraviolet (u.v.)-absorbing substances, as compared with the same organism grown in the presence of an optimal concentration ($8{\cdot}0 \times 10^{-10}$ M) of biotin. The concentration of acid-soluble u.v.-absorbing substances in the biotin-deficient yeast was higher, irrespective of the nature of the extracting acid (0·2 N-perchloric acid, 5 % (w/v) and 10 % (w/v) trichloroacetic acid, or 5 % (v/v) *n*-butanol in M/15 KH_2PO_4). Raising the temperature of extraction from 3° to 21° or 30° had little or no effect on the amounts of these u.v.-absorbing substances extracted. Analyses of the nucleotides and nucleobases in the yeast RNA showed these to have a ratio of purine:pyrimidine bases of 1·00–1·15, with the exception of the RNA from 5-day cultures of biotin-deficient yeast which had a slightly but consistently higher ratio. The significance of these results is discussed in relation to the metabolic function of biotin.

INTRODUCTION

Biotin has for some time been recognized as a growth factor for micro-organisms, but, although the metabolic roles of many other vitamins and growth factors have been elucidated, no specific function for biotin in the metabolism of micro-organisms has as yet been unequivocally established. Two main lines of study have been pursued in an attempt to gain information on the role of biotin in microbial metabolism. Growth of certain biotin-requiring micro-organisms in media containing suboptimal concentrations of biotin has been shown to be accompanied by the appearance in the medium of biosynthetic intermediates, the further metabolism of which is inhibited under conditions of biotin deficiency. Accumulation of an aromatic amine, 5-amino-imidazole riboside (Chamberlain, Cutts & Rainbow, 1952; Chamberlain & Rainbow, 1954; Moat, Wilkins & Friedman, 1956) and of hypoxanthine (Chamberlain & Rainbow, 1954) by *Saccharomyces cerevisiae* is the result of an impairment in the ability of the biotin-deficient yeast to complete the synthesis of purines. The inability to synthesize adequate quantities of purine under these conditions is also thought to cause a derangement in the synthesis of pyridine nucleotides by *S. cerevisiae*, which is manifested in the accumulation of

* Present address: Central Drug Research Institute, Lucknow, U.P. India.

nicotinic acid and nicotinic acid adenine dinucleotide in the culture medium (Rose, 1960*a*, *b*), while the same metabolic disturbance is probably responsible for the subnormal contents of adenosine triphosphate (ATP) and coenzyme A in biotin-deficient *Piricularia oryzae* (Katsuki, 1959*a*) and *Bacillus macerans* (Katsuki, 1959*b*).

Several workers have searched for a more specific locus of biotin function and have been able to show that, in certain biotin-deficient micro-organisms, the activity of some enzymes is markedly depressed. For example, a deficiency of biotin affects adversely the decarboxylation of oxaloacetate in *Lactobacillus arabinosus* (Lardy, Potter & Elvehjem, 1947), oxalosuccinate carboxylation in *Escherichia coli* (Shive & Rogers, 1947) and succinate decarboxylation in *Propionibacterium pentosaceum* (Delwiche, 1950). Biotin has also been shown to be essential for the activity of certain enzymes involved in amino acid metabolism, including those responsible for the aspartate to α-ketoglutarate transformation (Rossi, Rossi & Rossi, 1957), and for the deamination of aspartic acid (Lichstein & Umbreit, 1947), of threonine (Lichstein & Christman, 1948) and of serine (Nadkarni & Sreenivasan, 1957). Hexokinase activity in *Saccharomyces cerevisiae* (Strauss & Moat, 1958) and the ability to convert ornithine and carbamyl phosphate into citrulline in *Streptococcus lactis* (Estes, Ravel & Shive, 1956) have also been reported to be decreased under conditions of biotin deficiency.

However, the function of biotin in these biotin-dependent enzyme reactions has not yet been established. The activity of some of the enzymes in cell-free preparations is known to be stimulated on adding biotin, but a coenzymic role for the vitamin has not been demonstrated directly. Indirect evidence, such as the demonstration of a correlation between the activity of enzyme preparations and the content of bound biotin, is taken by some workers (e.g. Lichstein, 1955) to indicate a possible coenzymic role. There is, however, no evidence to show that the bound forms of biotin which have been isolated (such as ϵ-N-biotinyl-L-lysine, 'biocytin') are coenzymic forms of the vitamin (Wright *et al.* 1952). With other biotin-dependent enzyme systems no stimulation of activity occurs on adding biotin to the cell-free preparations, and this has led some workers to ascribe an indirect role for biotin, probably in enzyme synthesis. Sund, Ravel & Shive (1958), for example, did not obtain any immediate increase in activity of a preparation of the ornithine→citrulline enzyme from biotin-deficient *Streptococcus lactis* on adding biotin, and concluded that biotin is probably concerned in synthesis of the enzyme. Further evidence to support the view that biotin is concerned only indirectly in the activity of this enzyme comes from more recent data (Ravel, Grona, Humphreys & Shive, 1959), which showed that purified preparations of the enzyme contained less biotin than did the original cell-free extract. Similarly, Chambers & Delwiche (1954), as a result of their studies on the function of biotin in *Propionibacterium pentosaceum*, suggested that the vitamin functions in the synthesis of the coenzyme or apoenzyme concerned in the carboxylation of succinate.

The results from both of these lines of study suggest, therefore, that biotin is concerned in protein synthesis, either via the synthesis of purines or in the formation of specific enzymes. The work reported in this paper was carried out in order to examine the effect of biotin deficiency on the synthesis of nucleic acids and total protein in *Saccharomyces cerevisiae*. The results show that, under these conditions, synthesis of both of these groups of substances was impaired.

METHODS

Organism. The strain of *Saccharomyces cerevisiae* (Fleischmann) used was obtained from the Division of Applied Biology, National Research Council of Canada, Ottawa, and was maintained on slopes of malt wort agar: 10 % (w/v) spray-dried malt extract ('Muntona', Munton & Fison Ltd., Stowmarket, Suffolk) +2 % (w/v) agar. Cultures were stored at 3°.

Experimental cultures. The chemically defined medium of Rose & Nickerson (1956) was used. Portions of the medium (100 ml.), containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of biotin, were dispensed into 350 ml. conical flasks, which were plugged and sterilized by autoclaving momentarily at 10 lb./sq.in. The medium was inoculated by the procedure described by Rose (1960*b*), and cultures were incubated statically at 25°. Growth was measured turbidimetrically by determining the optical density of a portion (6 ml.) of culture in the Hilger 'Spekker' absorptiometer (model H 760), using neutral green-grey H 508 filters and a water blank. Optical density measurements were related to dry weight of yeast by a calibration curve.

Nucleic acid estimations. Yeast grown in media containing either an optimal or a suboptimal concentration of biotin was washed three times with M/15 KH_2PO_4, (pH 4·5), and triplicate 3 mg. portions of the crop were taken for nucleic acid estimations. The pellet of yeast was extracted twice, in 15 ml. tapered centrifuge tubes, with 2·0 ml. portions of 0·2 N-perchloric acid at room temperature to remove acid-soluble ultraviolet (u.v.)-absorbing substances. The extracts were pooled, neutralized with N-NaOH, and made up to 5·0 ml. with M/15 KH_2PO_4; the optical density of this extract was measured at 260 mμ, with the Unicam S.P. 500 quartz spectrophotometer, and the reading taken as a measure of the acid-soluble u.v.-absorbing substances in the yeast. The yeast pellet was then extracted twice with 3 ml. of a boiling mixture of 95 % (v/v) ethanol in water (3 vol.)+ether (1 vol.) for 2 min. to extract lipids, and the extracts rejected. The ribonucleic acid (RNA) in the residue was hydrolysed to acid-soluble nucleotides by suspending the material in 2·0 ml. N-NaOH for 1 hr. at room temperature (Schmidt & Thannhauser, 1945; Bonar & Duggan, 1955), after which perchloric acid (N) was added to a concentration of 0·2 N. The supernatant fluid containing the soluble RNA nucleotides was separated from the precipitate of deoxyribonucleic acid (DNA) and protein, which was then washed twice with 1·0 ml. portions of 0·2 N perchloric acid, and the washings combined with the RNA extract. The combined volume was neutralized with N-NaOH, made to 10·0 ml. with M/15 KH_2PO_4, and the optical density at 260 mμ taken as a measure of the RNA content of the yeast.

The residue of DNA and protein was suspended in 2·0 ml. N-perchloric acid, and held at 90° for 15 min. This hydrolysed the DNA to acid-soluble nucleotides, which were removed in the supernatant fluid. Extracts were made up to 3·0 ml. with N-perchloric acid, and the optical density of the solution at 260 mμ taken as a measure of the DNA content of the yeast.

Protein estimations. Protein in the residue remaining after the nucleic acids had been extracted was determined by the conventional micro-Kjeldahl technique (Markham, 1942) with a mercuric oxide catalyst (Miller & Houghton, 1945). Protein contents are expressed as mg. Kjeldahl nitrogen/3 mg. dry weight yeast.

Analysis of ribonucleic acids. For the electrophoretic separation of ribomononucleotides, the RNA extract from 40 mg. dry weight of yeast was adjusted to pH 4·0 by careful addition of 10·0 N-KOH, and potassium perchlorate removed by centrifugation (Davidson & Smellie, 1952). Portions (4·0 mg.) of a commercial preparation of yeast RNA (L. Light and Co. Ltd. Colnbrook, Buckinghamshire), which was used as a control, were dissolved in 1·0 ml. of 0·3 N-KOH, incubated at 37° for 18 hr., and the supernatant fluid removed by centrifugation after neutralization with 9·2 N-perchloric acid. The supernatant liquid was then adjusted to pH 4·0 with 10·0 N-KOH, and potassium perchlorate removed by centrifugation. Samples of the solutions (300–500 μl.) were applied as a short band (1·5–2·0 cm.) about 15 cm. from the end of a strip of Whatman no. 3 MM paper (57 cm. × 10 cm.). The paper was soaked in 0·02 M-citrate buffer (pH 3·5), and a potential gradient of 21 V./cm. applied for 7 hr. After removal, the paper was dried with a hair dryer, examined under u.v. radiation (Hanovia 'Chromatolite'), and the positions of the nucleotide spots marked with a pencil. The areas of paper containing the spots were cut out, and the nucleotides eluted from the shredded paper by soaking in 0·01 N-HCl overnight at 37° in a stoppered test tube. The eluate was centrifuged to remove cellulose fibres. The optical density of the eluate was then measured at the appropriate wavelength (adenylic acid, 260 mμ; guanylic acid, 260 mμ (Volkin & Carter, 1951); cytidylic acid, 278 mμ; uridylic acid, 262 mμ (Ploeser & Loring, 1949)), and the molar concentrations of the nucleotides in the eluates calculated from the optical density measurements by using the appropriate millimolar extinction coefficients. Controls of adenylic acid, guanylic acid, cytidylic acid and uridylic acid were run with each electrophoretogram.

Ribonucleobases were obtained by perchloric acid oxidation of the nucleotides. The extract of RNA mononucleotides from 40 mg. dry weight yeast was acidified to pH 5 and evaporated to dryness on a boiling water bath. This was followed by the addition of 0·2 ml. of 12·0 N perchloric acid, after which the solution was heated on the boiling water bath for 70 min. On cooling, 0·2 ml. distilled water was added, and the perchloric acid neutralized by addition of 10·0 N-KOH. The supernatant liquid was then acidified with pure HCl to 2·0 N-HCl, heated in a boiling water bath for a further 5 min. and cooled, after which the precipitate of potassium perchlorate and carbon was removed by centrifugation. This precipitate was washed with 0·1 N-HCl and the washings combined with the original extract. This was then applied as a band (1·0–1·5 cm.) on Whatman no. 3 MM paper, and examined by descending chromatography, using a solvent of *iso*propanol + conc. HCl + water (68 + 16·4 + 15·6; Wyatt, 1951). The paper was irrigated for 40 hr. at room temperature, dried, and examined beneath u.v. radiation. The nucleobases appeared as discrete absorbing spots, the guanine spot being easily distinguished by its bluish tinge. The appropriate areas of paper were cut out, and the nucleobases eluted from the shredded paper by soaking overnight at 37° in 0·10 N-HCl. The optical densities of the eluates were measured at the appropriate wavelengths (adenine, 260 mμ; guanine, 250 mμ; cytosine, 275 mμ; uracil, 260 mμ; Wyatt, 1951), and the molar concentrations of nucleobases calculated by using the appropriate millimolar extinction coefficients. Controls of adenine, guanine, cytosine and uracil were run with each chromatogram.

RESULTS

Effect of biotin deficiency on the concentrations of nucleic acids, protein and acid-soluble u.v.-absorbing substances in yeast

Cultures of the yeast, grown in media containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) concentration of biotin, were removed at intervals and, after growth had been measured, the yeast was washed and analysed for DNA, RNA, protein and acid-soluble u.v.-absorbing substances. The results are shown in Figs. 1 and 2. Under conditions of biotin deficiency, growth of the yeast was restricted and, after *c.* 120 hr. of incubation, the biotin-deficient yeast was coloured pink instead of the usual creamy-white (Chamberlain *et al.* 1952). This restriction in growth and change in colour of the yeast was accompanied by marked changes in

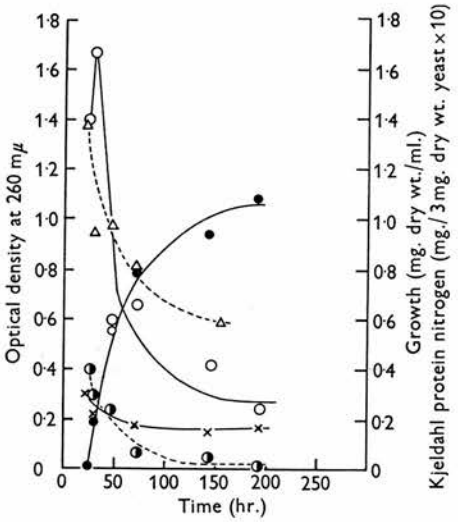

Fig. 1

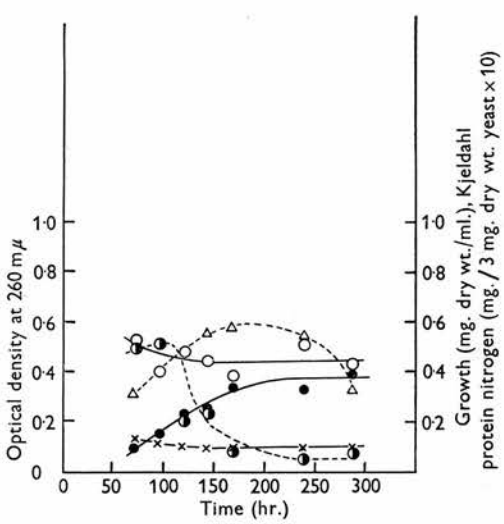

Fig. 2

Figs. 1, 2. Effect of incubation time on growth (●—●, mg. dry wt./ml.) and on the concentrations of DNA (×—×), RNA (○—○), acid-soluble u.v.-absorbing substances (◑—◑) and Kjeldahl protein nitrogen (△—△, mg./3 mg. dry wt. yeast × 10) in yeast grown in media containing either an optimal ($8{\cdot}0 \times 10^{-10}$ M) (Fig. 1) or a suboptimal ($0{\cdot}4 \times 10^{-10}$ M) (Fig. 2) concentration of biotin. Analyses were conducted on triplicate 3·0 mg. portions of yeast. Concentrations of DNA, RNA and acid-soluble u.v.-absorbing substances are expressed as the optical densities at 260 mμ of extracts from the yeast made up to 3·0, 10·0 and 5·0 ml. respectively with M/15 KH_2PO_4.

the concentrations of DNA, RNA, protein and acid-soluble u.v.-absorbing substances. After an initial slight decrease, the concentrations of DNA in both types of yeast remained constant throughout the period of growth, although under conditions of biotin deficiency, the concentration was significantly lower than in the yeast (biotin-optimal) grown in medium containing an optimal concentration of biotin. The sequence of changes observed in the RNA content of the biotin-optimal yeast was similar to that previously reported by other workers (Di Carlo & Schultz, 1948). In the biotin-deficient yeast, however, the concentration of RNA was, by comparison, low and remained so during the observed period of growth. The

concentration of Kjeldahl protein-nitrogen in the biotin-optimal yeast was highest during the very early stages of the exponential phase of growth, but declined steadily as the culture aged. In the biotin-deficient yeast, the protein nitrogen content increased up to 160 hr., when it was approximately half of that in exponential phase biotin-optimal yeast, but, thereafter, gradually declined. The biotin-optimal yeast contained an appreciable amount of acid-soluble u.v.-absorbing substances during the early stages of the exponential phase of growth but on further incubation the concentration declined rapidly and, at the end of the exponential phase, had become extremely small. The biotin-deficient yeast contained significantly higher concentrations of these substances during the early stages of growth although, after *c.* 200 hr., this concentration too had decreased to a low value.

Concentration of acid-soluble u.v.-absorbing substances and stability of RNA in biotin-deficient yeast

The comparatively high concentration of acid-soluble u.v.-absorbing substances in the biotin-deficient yeast during the early stages of growth was obviously of interest in relation to the inability of the yeast to synthesize normal amounts of RNA under conditions of biotin deficiency. It was possible that these u.v.-absorbing substances arose as a result of the breakdown of RNA in the biotin-deficient yeast during extraction with perchloric acid; alternatively, they may have represented purine- and pyrimidine-containing substances that had failed to be polymerized into DNA and RNA. A study was therefore made of the effect of using various acid solutions, including 0·2 N and 1·0 N-perchloric acid, 5 % (w/v) and 10 % (w/v) trichloroacetic acid, and 5 % (v/v) *n*-butanol in M/15 KH_2PO_4 (pH 4·5), to extract these u.v.-absorbing substances from 120 hr. biotin-deficient yeast and from exponential phase (40 hr.) and stationary phase (120 hr.) biotin-optimal yeast. Triplicate 3 mg. portions of washed yeast were taken, and were extracted five or, when necessary, more times with 4·0 ml. portions of the extracting solution at 3° for 5 min. After centrifugation, the supernatant liquid was decanted, adjusted to pH 4·5, and made up to 5·0 ml. with M/15 KH_2PO_4. The optical densities of these extracts were then measured at 260 mμ, with a blank of the appropriate reagent. Removal of the u.v.-absorbing substances was usually complete in five extractions, although complete removal from biotin-deficient yeast with the 5 % aqueous butanol required seven separate extractions. After all the u.v.-absorbing substances had been extracted, the residue was defatted and the RNA estimated in the usual way.

Perchloric acid (0·2 N) extracted all the u.v.-absorbing substances fairly rapidly from biotin-deficient yeast and from biotin-optimal yeast. Consistently larger amounts of u.v.-absorbing substances were extracted from biotin-deficient yeast than from either exponentially growing or stationary phase yeast grown in presence of optimal biotin (Fig. 3). Higher concentrations of perchloric acid (e.g. N) are known to hydrolyse RNA, and this is used as a means of extracting RNA from tissues (Ogur & Rosen, 1950). However, it would seem from the data shown in Fig. 4 that the RNA in biotin-deficient yeast was hydrolysed more quickly by N-perchloric acid as compared with RNA in biotin-optimal yeast.

Many workers have recommended the use of trichloroacetic acid for the extraction

of u.v.-absorbing substances before estimation of nucleic acids in tissues (Davidson, Frazer & Hutchinson, 1951; Schmidt & Thannhauser, 1945; Schneider, 1945). Trichloroacetic acid at concentrations of 5 % (w/v) or 10 % (w/v) rapidly extracted the bulk of the acid-soluble u.v.-absorbing substances from the biotin-deficient yeast and from the biotin-optimal yeast. The amounts extracted from biotin-deficient yeast again exceeded those from non-deficient yeast (Fig. 5). Little difference was

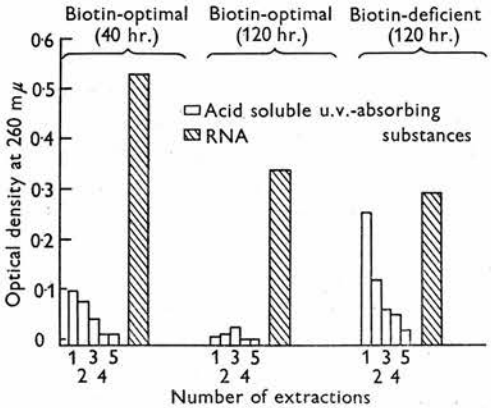

Fig. 3

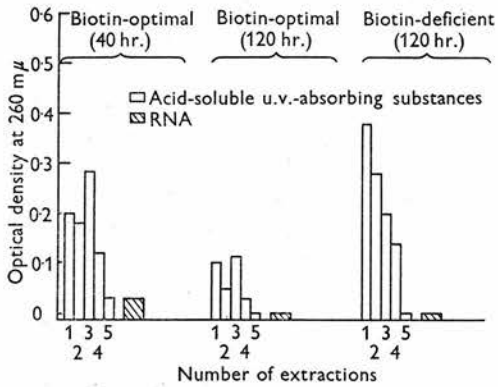

Fig. 4

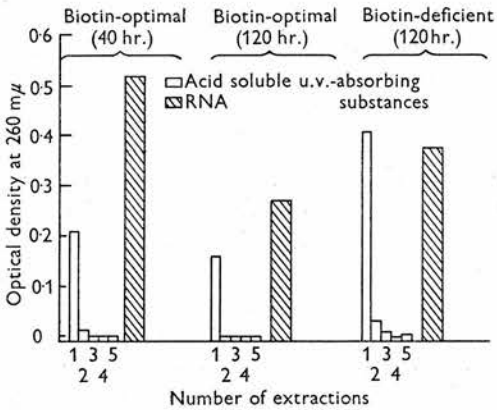

Fig. 5

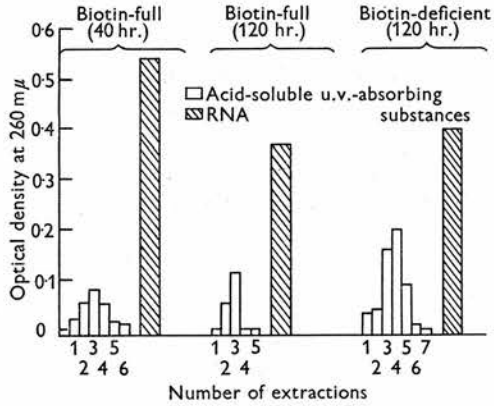

Fig. 6

Figs. 3–6. Extraction of acid-soluble u.v.-absorbing substances from 3·0 mg. portions of biotin-optimal (40 and 120 hr.) and biotin-deficient (120 hr.) yeast with separate 4·0 ml. portions of 0·2N-$HClO_4$ (Fig. 3), 1·0N$HClO_4$ (Fig. 4), 5 % (w/v) trichloroacetic acid (Fig. 5) or 5 % (v/v) *n*-butanol in M/15KH_2PO_4 (Fig. 6). Extracts were adjusted to pH 4·5, made up to 5·0 ml. with M/15 KH_2PO_4, and the optical densities measured at 260 mμ. RNA was extracted as described under Methods.

observed between the amounts extracted by 5 % and 10 % trichloroacetic acid, although a slightly decreased content of RNA in biotin-deficient yeast that had been extracted with 10 % trichloroacetic acid suggested that some of the nucleic acid may have been hydrolysed during extraction.

Extraction of u.v.-absorbing substances by aqueous *n*-butanol was used by Mitchell & Moyle (1951) in their studies on the chemical anatomy of *Staphylococcus*

aureus (*Micrococcus pyogenes*). This reagent is far milder than either perchloric acid or trichloroacetic acid, and was used in an attempt to minimize possible hydrolytic breakdown of RNA during extraction. Concentrations of *n*-butanol up to 4% (v/v) in M/15 KH_2PO_4 failed to extract detectable amounts of u.v.-absorbing substances from the yeast. But by using a concentration of 5% (v/v) butanol in M/15 KH_2PO_4, the u.v.-absorbing substances were extracted, although at least seven separate extractions were required for complete removal of these substances from biotin-deficient yeast (Fig. 6).

Effect of temperature. Further information about the stability of the RNA in biotin-deficient yeast during extraction of the acid-soluble u.v.-absorbing substances was obtained when these extractions were carried out at 21° or 30° instead of at 3°. The results obtained showed that elevation of the temperature had no significant effect on the amounts of u.v.-absorbing substances and of RNA extracted, as compared with the amounts extracted at the lower temperature. Extraction at these elevated temperatures did not affect the amounts of acid-soluble u.v.-absorbing substances extracted from biotin-optimal yeast. Spectrophotometric examination of the various extracts of acid-soluble u.v.-absorbing substances revealed that, in all instances, these showed maximum u.v. absorption at or very close to 260 mμ.

Effect of biotin deficiency on the nucleotide and nucleobase compositions of the yeast RNA

The nucleotide and nucleobase compositions were determined on the RNA extracted from 40 mg. dry wt. yeast. The yeast was extracted with 10 ml. portions of 5% (w/v) trichloroacetic acid at 3° until the acid-soluble u.v.-absorbing substances had been completely removed; this required five to eight separate extrac-

Table 1. *Molar concentrations of adenylic, guanylic, cytidylic and uridylic acids (based on adenylic acid = 10) in a commercial yeast RNA and in extracts of RNA from* Saccharomyces cerevisiae *grown in media containing an optimal* ($8{\cdot}0 \times 10^{-10}$ M) *or a suboptimal* ($0{\cdot}4 \times 10^{-10}$ M) *concentration of biotin*

Source	Age of culture (hr.)	Molar concentration				Ratio: purine/ pyrimidine
		Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	
Commercial yeast RNA	—	10·0	12·64	7·73	12·20	1·13
Biotin-optimal yeast	40	10·0	11·12	8·00	11·31	1·11
	96	10·0	10·6	8·20	11·60	1·03
Biotin-deficient yeast	120	10·0	11·90	7·40	9·76	1·28
	168	10·0	12·70	8·19	12·08	1·12

tions. The tissue was defatted by extracting twice with 10 ml. portions of a boiling mixture of 95% (v/v) ethanol in water (3 vol.)+ether (1 vol.), and the residue treated with 2·0 ml. 0·3 N-KOH for 18 hr. at 37° (Davidson & Smellie, 1952) to hydrolyse polyribonucleotides to soluble mononucleotides. Shorter periods of incubation were tried and, although these were sufficient to allow for the hydrolysis of RNA to acid-soluble nucleotides as detected spectrophotometrically, nevertheless it was shown electrophoretically that incubation for 18 hr. at 37° was necessary

to obtain complete hydrolysis to the mononucleotides. The nucleotide and nucleobase compositions of the RNA extract were then determined as described under Methods.

The data in Table 1 show the molar concentrations (with adenylic acid expressed as 10) of RNA nucleotides in a sample of commercial yeast RNA and in the RNA from *Saccharomyces cerevisiae* grown in media containing either an optimal or a suboptimal concentration of biotin. These results show the molar ratio of purine to pyrimidine nucleotides ranged from 1·00 to 1·15 in the commercial yeast RNA

Table 2. *Molar concentrations of adenine, guanine, cytosine and uracil (based on adenine = 10) in a commercial yeast RNA and in extracts of RNA from* Saccharomyces cerevisiae *grown in media containing an optimal* ($8{\cdot}0 \times 10^{-10}$ M) *or a suboptimal* ($0{\cdot}4 \times 10^{-10}$ M) *concentration of biotin*

Source	Age of culture (hr.)	Molar concentration				Ratio: purine/ pyrimidine
		Adenine	Guanine	Cytosine	Uracil	
Commercial yeast RNA	—	10·0	10·10	8·07	11·03	1·05
Biotin-optimal yeast	40	10·0	11·36	7·91	11·50	1·10
	96	10·0	11·14	8·50	11·40	1·04
Biotin-deficient yeast	120	10·0	11·60	7·56	10·10	1·23
	168	10·0	11·60	9·10	11·90	1·02

and in biotin-optimal yeast during the exponential and stationary phases of growth. In biotin-deficient yeast from 120 hr. cultures, the ratio was slightly but consistently higher, the average value obtained being *c.* 1·28; but in yeast from 7-day biotin-deficient cultures, the ratio had decreased to within the range 1·00–1·15. Closely similar results were obtained when the ratio of purine to pyrimidine bases in the yeast RNA was determined. As shown in Table 2, this ratio was in the range 1·00–1·10 in all of the samples of RNA studied, with the exception of that obtained from 120 hr. cultures of biotin-deficient yeast in which it averaged 1·23.

DISCUSSION

Since purine- and pyrimidine-containing nucleotides (e.g. ATP, DPN, coenzyme A) and polynucleotides (nucleic acids) are essential components of all living cells, it is to be expected that any metabolic stress which causes a derangement in the biosynthetic processes leading to the formation of purines or pyrimidines will result in the decreased synthesis of nucleotides and nucleic acids. Although the presence of diminished amounts of total purine in biotin-deficient micro-organisms has not been demonstrated directly, several workers have obtained evidence that biotin is concerned in the synthesis of these nitrogenous bases. The effects of this biotin-conditioned purine deficiency on the synthesis of certain nucleotides in biotin-requiring micro-organisms has already been reported. Thus, Katsuki (1959*a*, *b*) has shown that biotin-deficient *Piricularia oryzae* and *Bacillus macerans* contained diminished amounts of ATP, DNP and coenzyme A, and Rose (1960*b*) reported that the excretion of nicotinic acid and nicotinic acid adenine dinucleotide, two biosynthetic precursors of pyridine nucleotides which appear in the culture medium during growth of *Saccharomyces cerevisiae* under conditions of biotin deficiency, is suppressed on adding adenine to the medium. The results obtained in the present

investigation showed that biotin deficiency during growth of a strain of *S. cerevisiae* had a profound effect also on the synthesis of nucleic acids. It was somewhat surprising to discover that, during the early stages of growth, RNA in the biotin-deficient yeast contained an abnormally high content of purine, in view of the adverse effect of biotin deficiency on purine biosynthesis. There was further evidence of a difference between the RNA from 5-day biotin-deficient cultures and that from other cultures, in that the former was more readily hydrolysed by N-perchloric acid. This may indicate a certain instability in structure, a reflexion perhaps of the slightly abnormal base ratio. It is important to note, however, that the RNA extracted from the yeast was heterogeneous and consisted of a mixture of ribosomal, soluble and nuclear RNA. The slight difference in the overall base ratio of the mixture might then be caused by a more significant variation in the base ratio of one of these RNA fractions.

This diminution in the amounts of nucleic acids synthesized under conditions of biotin deficiency was accompanied, during the early stages of growth, by a significant increase in the concentration of intracellular acid-soluble u.v.-absorbing substances which, since they absorbed maximally at approximately 260 mμ, were taken to be purine- and pyrimidine-containing substances. The substances were detected initially in extracts made with the strong acids perchloric and trichloroacetic acids, and it was possible that they represented products from the acid degradation of RNA and DNA. When it was discovered, however, that these u.v.-absorbing substances were also extracted, albeit more slowly, with aqueous *n*-butanol at pH 4·5, a much less drastic reagent, then it was assumed that they were present in the yeast in the soluble state and that they did not represent artefacts of extraction. Some evidence to support this contention came from the discovery that the amounts of u.v.-absorbing substances extracted with acid did not increase significantly when the temperature of extraction was raised from 3° to 21° or 30°, as might have been expected had they arisen as the result of hydrolysis of RNA. No analyses of the composition of this acid-soluble fraction were made in the present study, so it is not known what type of purine- or pyrimidine-containing compounds were present. But at least two -u.v.-absorbing purine precursors, 5-amino-imidazole riboside (Moat *et al.* 1956; Lones, Rainbow & Woodward, 1958) and hypoxanthine (Chamberlain & Rainbow, 1954), are known to be excreted by *Saccharomyces cerevisiae* growing under conditions of biotin deficiency, so that it is likely that these also accumulate in the cells. It is possible too, that ribonucleotides are present in this fraction since these are known to accumulate intracellularly under conditions of decreased RNA synthesis. This has been demonstrated, for example, with a strain of *Escherichia coli* following addition to the culture of the purine analogue 6-azauracil (Skoda & Sorm, 1958).

The role of RNA in protein synthesis is well known, and there is also some reason for believing that protein synthesis is essential for the synthesis of RNA. The results obtained in the present study showed that, under conditions of biotin deficiency, synthesis of total protein by *Saccharomyces cerevisiae* was markedly diminished, and it is possible that this was a result, at least in part, of the decrease in the amount of RNA synthesized and of the formation of possibly abnormal RNA. But for protein synthesis to take place, it is also essential to have in the cell an adequate reservoir or pool of amino acids as well as sufficient energy, in the

form of ATP, to activate these amino acids. It is probable, however, that neither of these requirements is met in biotin-deficient yeast, for, not only does biotin-deficient *S. cerevisiae* contain reduced amounts of ATP (Dr M. H. Briggs, personal communication), but it has also been found to contain decreased concentrations of water-soluble ninhydrin-positive substances (Mr A. L. S. Munro, unpublished observations) as compared with biotin-optimal yeast. It would appear, therefore, that there are several metabolic deficiencies contributing towards this overall reduction in protein synthesis.

The diminution in the amount of protein synthesized in biotin-deficient yeast must clearly affect the enzymic activities of the yeast. It is possible that the production of only certain enzymes is affected under conditions of biotin deficiency, which would explain why only a limited number of enzymic activities have been reported to be impaired in yeast grown under this metabolic stress. On the other hand, biotin deficiency may have a non-specific effect on protein synthesis, with the result that production of all of the enzymes in the cell is decreased to about the same extent. Then the deficiency might only be observed in those metabolic reactions for which the enzymes are present in rate-limiting concentrations.

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BIOCHIMICA et BIOPHYSICA ACTA

Changes in the polysaccharide composition of yeast resulting from biotin deficiency

Growth of biotin-requiring micro-organisms under conditions of biotin deficiency has been shown to bring about certain changes in the chemical composition of the cells. Biotin-deficient *Saccharomyces cerevisiae* contains less DNA, RNA and total protein[1] and has a slightly smaller intracellular amino acid pool[2] as compared with exponentially-growing yeast; under conditions of biotin deficiency, however, the yeast accumulates increased amounts of acid-soluble ultraviolet-absorbing substances. The decrease in the amount of cellular protein formed in biotin-deficient yeast is accompanied by a diminished activity of several enzymes, many of these enzymes being concerned with the metabolic transfer of carbon dioxide[3]. Studies in this laboratory have now revealed that, under conditions of biotin deficiency, the polysaccharide composition of yeast is also changed.

The strain of *Saccharomyces cerevisiae* (Fleischmann) used in these studies was grown in a glucose–salts–vitamins medium[4] containing a suboptimal concentration ($0.4 \cdot 10^{-10}$ *M*) of D-biotin, using the experimental conditions described by AHMAD, ROSE AND GARG[1]. Addition of L- or DL-aspartic acid ($2.0 \cdot 10^{-3}$ *M*) to this biotin-deficient medium brought about a slight increase in growth and also caused the yeast to grow in clumps which adhered to the walls of the culture flask. When Difco vitamin-free casamino acids (2.0 mg/ml) was included in the biotin-deficient medium, the cells again grew in clumps, but the increase in growth was greater than in biotin-deficient medium containing aspartate alone. Microscopic examination revealed that, during growth of the yeast in these amino acid-containing, biotin-deficient media, the daughter cells formed during budding failed to separate from the parent cells, and so gave rise to large aggregates of cells (Fig. 1). Dry-weight determinations showed that there was no significant change in the turbidity–dry weight relationship in suspensions of the clumped yeast that had been dispersed by shaking, as compared with suspensions of yeast grown in biotin-optimal media or in amino acid-free biotin-deficient media. Addition of oleic acid (100 μg/ml), adenine or adenosine ($1.0 \cdot 10^{-3}$ *M*) to biotin-deficient medium has been shown to stimulate yeast growth but, when these compounds were incorporated into the amino acid-containing, biotin-deficient media either singly or together, they did not prevent formation of cell aggregates. Yeast grown in aspartate-free biotin-deficient medium or in biotin-optimal ($8.0 \cdot 10^{-10}$ *M*) medium containing L-aspartic acid did not clump. All of the biotin-sparing compounds used in these studies were shown to be free of detectable amounts of biotin[6].

GHOSH *et al.*[7] reported the appearance of a similar type of cell aggregation during growth of *Saccharomyces carlsbergensis* under conditions of myoinositol deficiency, and showed that aggregation of the yeast under these conditions was accompanied by production of greater amounts of glucan than in inositol-optimal yeast. Experiments were therefore carried out to discover whether cell aggregation in *S. cerevisiae* during growth in aspartate-containing, biotin-deficient medium was also accompanied by changes in the amounts of polysaccharides produced. Quantitative fractionation of the yeast carbohydrates was carried out using the method described by CHUNG AND NICKERSON[8]. Total yeast carbohydrate and the carbohydrate contents of the individual fractions were estimated using the modification of the anthrone method described by HALL[9], but employing a 10-min heating at 100° as used by CHUNG AND NICKERSON[8],

Fig. 1. Photomicrograph showing the large aggregates of cells of *Saccharomyces cerevisiae* grown in a biotin-deficient medium containing L-aspartic acid (magnification × 675).

absorbancy readings at 540 mμ were related to carbohydrate content using a glucose standard curve. The results of this study are summarised in the data in Table I. These give the total carbohydrate content and the percentage compositions of carbohydrates extractable by water or 10% trichloroacetic acid, glycogen, mannan and glucan in yeast from 40-h cultures grown in a medium containing an optimal concentration ($8.0 \cdot 10^{-10}$ *M*) of biotin and from 5-day cultures grown in a medium containing a suboptimal concentration ($0.4 \cdot 10^{-10}$ *M*) of biotin in the presence and absence of L-aspartic acid ($2.0 \cdot 10^{-3}$ *M*). The purity of the glucan and mannan fractions from yeast grown in each of these media was confirmed by paper chromatographic analysis of acid hydrolysates of the fractions.

Yeast grown under conditions of biotin deficiency, but in the absence of aspartate, contained approximately the same amount of total carbohydrate as biotin-optimal yeast; however, when grown in biotin-deficient medium containing aspartate, the total carbohydrate content of the cells increased. The percentage compositions of water-extractable carbohydrates and glycogen were approximately the same in yeast grown in each of the three media. However, yeast grown in aspartate-free, biotin-deficient medium contained increased proportions of carbohydrates extractable by trichloroacetic acid and of glucan and diminished amounts of mannan as compared with bio-

tin-optimal yeast. When grown in a biotin-deficient medium containing L-aspartate, the yeast contained an even greater proportion of glucan and much less mannan than cells grown in the absence of aspartate; these changes were accompanied by a drop in the percentage composition of carbohydrates extractable by trichloroacetic acid.

The polysaccharides glucan and mannan have been shown to occur in the cell walls of Saccharomyces species[10,11], so that any change in the proportion of these two polysaccharides produced will presumably affect the physico-chemical properties of the yeast cell wall. The results reported in this paper, and those obtained by GHOSH *et al.*[7], show that aggregation of yeast cells in the amino acid-containing, biotin-deficient media or in inositol-deficient media is accompanied by an increase in the amount of glucan produced by the yeasts. However, with the experimental evidence at present available, there is no reason to believe that formation of cell aggregates is a direct consequence of the over-production of glucan. The marked decline in mannan production may well mean that there are insufficient amounts of this polysaccharide being produced for completion of cell division, and that this results in a change in the structure of the glucomannan-protein complexes which, as reported by KESSLER AND NICKERSON[11], are present in the cell walls of *S. cerevisiae*.

TABLE I

EFFECT OF BIOTIN CONCENTRATION AND L-ASPARTIC ACID ON THE CARBOHYDRATE COMPOSITION OF *Saccharomyces cerevisiae*

	Basal medium supplemented with		
	Optimal biotin *	*Suboptimal biotin* **	*Suboptimal biotin + L-aspartate* **
Carbohydrate	*Percentage total carbohydrate*		
Water-extractable	0.3	0.2	0.4
Extractable in 10% trichloroacetic acid	3.7	6.7	2.1
Glycogen	8.9	9.4	9.0
Mannan	42.0	31.7	19.1
Glucan	45.1	52.0	69.4
	µg "glucose equivalent"/mg dry wt. yeast		
Sum of fractions	238	252	305
	µg "glucose equivalent"/mg dry wt. yeast determined on whole cells		
Total carbohydrate	238	248	332

* 40-h cultures
** 5-day cultures

According to CHUNG AND NICKERSON[8], synthesis of glucan and mannan by *S. cerevisiae*, growing in glucose-containing media, proceeds via the formation of glucose and mannose 1-phosphates which, in turn, are formed from hexose 6-phosphates. Overproduction of glucan at the expense of mannan under the stress of biotin deficiency might then be explained by the development of a metabolic lesion at one or more steps during the synthesis of mannan from glucose phosphates. Aspartic acid is known to spare the growth-promoting action of biotin on micro-organisms[12,13], certain other amino acids having a similar though less marked effect[13]. Moreover,

growth of biotin-deficient *S. cerevisiae* in the presence of L-aspartic acid causes a marked restoration of RNA synthesis[4], so that the increased differences in the proportions of glucan and mannan in yeast grown under these conditions, compared with biotin-deficient yeast grown in the absence of aspartate, could be a reflection of the increased metabolic activity of the yeast; further evidence of this increased metabolic activity is seen in the decline in the percentage of carbohydrates extractable by trichloroacetic acid. It would seem, however, that growth in the presence of aspartate does not materially affect the lesion in the path to mannan synthesis. Since none of the biotin-sparing compounds had the ability, either singly or together, to prevent formation of cell aggregates in the amino acid-containing, biotin-deficient media, it must be presumed either that cell aggregation is a manifestation of some metabolic abnormality induced by the presence of aspartic acid and other amino acids, or that the synthesis of mannan in normal amounts involves certain metabolic reactions in which the biotin-sparing compounds tested cannot substitute for biotin.

Microbiology Laboratory,
Department of Applied Biochemistry,
Heriot-Watt College, Edinburgh (Great Britain)

J. L. DUNWELL
FAZAL AHMAD
A. H. ROSE

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